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Physiological Zoölogy

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FEEDING, DIGESTION, AND STARVATION IN AMOEBA PROTEUS (LEIDY)[1]

(Three figures)

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NUMEROUS observations on the process of feeding have been made by various investigators. Schaeffer (1916, 1917) made an intensive study of the subject and reviewed the literature to date. He concludes (1916*a*, p. 550): "Ameba is essentially a beast of prey." "A hungry ameba eats any organism it can get hold of. Protozoa of any size ranging from a small flagellate such as chilomonas, to a paramecium; rotifers, small entomostraca; diatoms and desmids, etc.; all are eaten providing only that they move slowly enough to allow of the formation of a food cup over them." He also concludes that it eats all sorts of inanimate particles, provided they are not poisonous, e.g. carmine, carbon, glass, uric acid, starch, globulin, pieces of meat, etc.; but that small flagellates form the larger part of the food of some species, e.g. *Amoeba proteus*, and diatoms and desmids of others, e.g. *Amoeba dubia*.

He holds that cups are always formed in the process of feeding, but that the size, the method of formation and the structure of the cups vary greatly in accord with the views of Kepner and Taliaferro (1913). Edwards (1925) maintains that under certain conditions

[1] Most of the observations considered in this paper were made in the Mount Desert Island Biological Laboratory and the Franz Theodore Stone Laboratory. We are much indebted to the directors of these laboratories for the excellent facilities put at our disposal.

food cups are formed in the absence of solid particles and that fluid is ingested in that way. Mast and Doyle (1934*a*) found that in solutions of albumin or in hypertonic solutions of balanced salts or of calcium gluconate numerous tubes filled with fluid are formed and that these later disintegrate and release their fluid content inside the organism. Schaeffer (1916, p. 546*a*) contends that *Amoeba proteus* sometimes forms two or even three food cups at the same time, and (1917, p. 249*a*) that if the organism it is feeding on is small, e.g. *Chilomonas*, it takes in several hundred a day. He maintains that the time that ingested organisms live in *Amoeba* and the time they are retained varies greatly. He says (1916, p. 549*a*): "A hungry ameba kills a chilomonas in a minute or less and coleps in two to five minutes"; but small flagellates sometimes live for more than an an hour, and globulin, gluten, fragments of meat, etc. "may be carried three or four days," while indigestible substances, e.g. glass and carmine, are usually retained only a few minutes.

Mast and Root (1916), Kepner and Whitlock (1921) and Beers (1924) observed that in feeding on large protozoa, e.g. *Paramecium* and *Frontonia*, *Amoeba proteus* sometimes pinches them in half. Mast and Root demonstrated that this process cannot be accomplished by the action of surface tension.

Schaeffer says that much water is taken in with the food under some conditions and very little under others, and that if much is taken in, it soon disappears while small irregular projections are thrown out from the inside wall of the vacuole toward the food and that granules of globulin in it decrease in size, but he gives no further information concerning changes in the vacuole.

Greenwood (1886, 1887) maintains that proteins are digested, but that starch is not and that digestion of fat is doubtful.

Dawson and Belkin (1928, 1929) injected various oils into *Amoeba proteus* and *Amoeba dubia;* they assert that codliver, olive, cottonseed, linseed, sperm, peanut, oxfoot, and oleic oils were digested in *Amoeba proteus* and all these, except oxfoot and oleic oils, in *Amoeba dubia;* but that the paraffin oil, nujol, was not digested in either.

We have in connection with the investigation of various processes made numerous observations on feeding in *Amoeba proteus*, and we

have devoted considerable time to the problems of digestion and starvation. The results obtained extend those obtained by others. They will be considered in the following pages.

METHOD OF FEEDING

Amoeba proteus was seen to feed only by means of food cups, but the cups seen varied very greatly in method of formation, structure, and size. The results obtained regarding this are in full accord with the conclusions reached by Kepner and Taliaferro (1913) and Schaeffer (1916). Usually only one organism is ingested in a food cup, but 13 specimens of *Chilomonas paramecium* were seen taken in one; 14 specimens of *Monas punctum* in another, and 3 specimens of *Colpidium colpoda* in still another. Two different species were also seen ingested in the same cup.

The specimens taken in a food cup are not always retained in the food vacuoles formed. Food vacuoles sometimes fuse to form larger vacuoles and sometimes divide as described below.

CONDITIONS WHICH FACILITATE FEEDING

Amoeba proteus does not feed when it is not attached to the substratum, therefore anything which facilitates attachment facilitates feeding. It was repeatedly observed that feeding increased greatly if amoebae were transferred from a solution in which attachment was weak to one in which it was strong, e.g. fresh Chalkley solution. It was also repeatedly observed that feeding increased if the amoebae were agitated. This was particularly marked in specimens transferred from ordinary culture dishes to slides. Starvation facilitates feeding but not if it continues until the amoebae no longer attach firmly. Specimens kept without food five to eight days usually do not feed again no matter how they are treated, although they often live ten days longer.

KIND OF FOOD

Amoeba proteus usually feeds exclusively on living organisms. It was never observed to take dead specimens. It sometimes ingests inanimate particles of various kinds but these doubtless have but

little if any value as food.[2] The results obtained in our observations indicate that its food usually consists very largely of *Chilomonas* and *Colpidium* but that under certain conditions it will ingest any animal or plant that is not too large or too active. This is in accord with Schaeffer's views.

CHOICE OF FOOD

Schaeffer (1917*a*, p. 250) says: "Amoeba is capable of exercising very nice discrimination in feeding between two particles of different composition—one digestible, the other not. . . . . The food cup in some way separates them so that the food particle comes to lie inside the food cup, while the other particle is actively pushed to the outside." We made numerous observations at various times on the organisms eaten by *Amoeba proteus* in cultures containing *Colpidium colpoda*, *Chilomonas paramecium*, and *Monas punctum* in various proportions. The results obtained indicated that it takes all of these species, that there is no discrimination between *Colpidium* and *Chilomonas*, but that it usually does not take *Monas* if *Colpidium* or *Chilomonas* is available. The results consequently indicate that it does not discriminate between *Chilomonas* and *Colpidium* but that it does discriminate between *Monas* and *Colpidium* or *Chilomonas*.

Monas punctum is a flagellate similar in size, form, and activity to *Chilomonas paramecium*. It consequently seemed desirable to extend the observations on discrimination between them. These observations were made in two ways:

1. Several amoebae were mounted in culture solution containing *Chilomonas* and *Monas* in approximately equal numbers. An amoeba which was feeding was then selected and observed continuously and the number of contacts it made with specimens of *Chilomonas* and *Monas* respectively in relation to the number eaten recorded. This was repeated with nine other amoebae. During the time of observation on these ten amoebae there were a total of 56 contacts with chilomonads and 66 contacts with monads. Thirty-four of the for-

[2] W. L. Doyle says that under some conditions *Amoeba proteus* freely ingests numerous bacteria in masses of zoöglea and that it can live on these substances for considerable periods of time (personal communication).

mer were surrounded by food cups but only 4 of the latter. Eleven of the former escaped and 3 of the latter, i.e. the 56 contacts with chilomonads resulted in ingestion of 23 while the 66 contacts with monads resulted in ingestion of only one.

2. Amoebae were starved until the food vacuoles were eliminated and then put into culture solution containing a known proportion of chilomonads and monads and left 30 minutes, after which they were killed with Lugol solution and the number of chilomonads and monads in them recorded.

With the ratio between *Chilomonas* and *Monas* 1:5.6 the 32 amoebae studied contained 67 chilomonads and 12 monads. With the ratio 1:3.4, 10 contained 15 chilomonads and 1 monad. With the ratio 1:0.7, 38 contained 587 chilomonads and 6 monads. These results show that when the chilomonads and the monads are approximately equal in number *Amoeba proteus* takes nearly one hundred times as many of the former as the latter and that the percentage of monads taken increases as the relative number in the culture increases. The results obtained by both methods consequently support the conclusion reached on the basis of superficial observation, namely, that *Amoeba proteus* discriminates between *Chilomonas paramecium* and *Monas punctum*. Whether this discrimination is on the basis of physical or of chemical difference between the two organisms is not known. The results obtained in the observations on the relation between contact and selection favors the latter. The fact that *Chilomonas* is much more readily digested than *Monas* seems to favor the latter also.

LENGTH OF LIFE IN THE FOOD VACUOLES

The time that organisms remain alive in the food vacuoles varies greatly with different species and also with different individuals of the same species. The time was ascertained for thirty chilomonads and two colpidia. It was found to vary from 3 to 18 minutes (average 9 minutes) for *Chilomonas* and from 5 to 14 minutes for *Colpidium*. Some species live much longer. *Monas punctum*, e.g. sometimes lives more than 3.5 hours. No detailed measurements were made in reference to this but living specimens were found in amoebae 3.5 hours after they had been isolated without food.

SIZE AND CONTENT OF FOOD VACUOLES AND PHYSICAL CHANGES IN THEM

Each food vacuole immediately after it is formed contains a relatively large amount of water and one or more organisms. The water decreases rapidly in amount by diffusion into the cytoplasm and sometimes also by division into two parts, one containing only water and the other the food and water. If the vacuole has more than one organism it usually divides into as many parts as there are organisms. If the organisms are small, e.g. *Chilomonas*, there is no further division but if they are large it frequently divides again. Vacuoles containing *Colpidium* were repeatedly seen to divide into two parts, each containing approximately one-half of the organism and a few were seen to divide into three or four parts. Vacuoles containing *Monas* were never seen to divide.

A considerable number of amoebae which contained extraordinarily large food vacuoles were found at various times, especially in old cultures (Fig. 1). By crushing these amoebae it was demonstrated that these vacuoles contained *Monas punctum*. The monads in several specimens which had been crushed were counted. In one which contained a very large vacuole and a few small ones, 1,284 were found (Fig. 1*A*); all but about 8 of these had been in the large vacuole. Practically all of the monads were inactive when the amoeba was crushed, but the following morning nearly all were active. There was no indication of digestion in any of them.

These large vacuoles are doubtless formed by fusion of small ones, for vacuoles containing one or several monads each were, a number of times, seen to fuse. This was also observed to occur in vacuoles containing mold spores but it was never observed in vacuoles containing either *Chilomonas* or *Colpidium*. It appears to be associated with organisms which are not readily or not at all digested.

AMOUNT OF FOOD

No detailed observations were made on the amount of food consumed by *Amoeba*, but some idea as to this can be obtained by calculation based on the number of organisms usually found in it and the time it takes to digest them.

Amoebae which are in good condition and are feeding chiefly on

Chilomonas usually contain approximately 50 vacuoles each. It requires 12 to 24 hours to digest a chilomonad. An amoeba feeding on *Chilomonas*, therefore, probably consumes between fifty and one hundred a day.

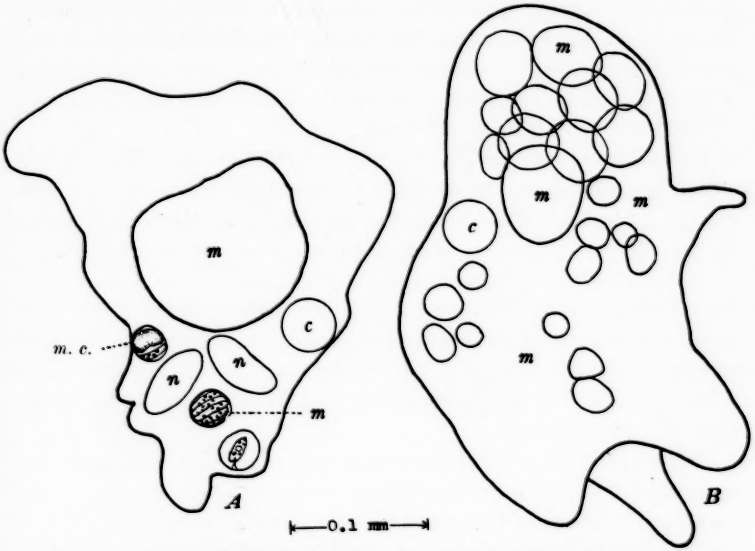

FIG. 1.—Camera outlines of *Amoeba proteus* containing giant food vacuoles filled with *Monas punctum*. *n*, nucleus; *c*, contractile vacuole; *m*, food vacuoles containing monads; *m.c*, a food vacuole containing a monad and a colpidium; *mm*, projected scale.

Specimen *B* contained a total of 1,013 monads and specimen *A* a total of 1,284, nearly all of which were in the large vacuole.

DIGESTION IN AMOEBA PROTEUS

Chilomonas paramecium

Chilomonas usually contains relatively large quantities of starch and fat, a considerable number of small spherical bodies which stain deep red with neutral red, two ellipsoid bodies which stain purple with neutral red, a nucleus, and various other constituents of the cytoplasm. The small spherical bodies will be called neutral red droplets or substance and the ellipsoid bodies, ellipsoids.

Three different methods were used in making observations on digestion of *Chilomonas*.

1. A small quantity of neutral red was added to culture solution containing numerous amoebae and chilomonads, and left until the amoebae had food vacuoles containing chilomonads in all stages of digestion. Some were then mounted under a cover-glass and much flattened by carefully removing some of the solution so as not to injure them. They were then thoroughly examined under an oil immersion objective. Similar observations were also made on unstained specimens and on specimens which had been killed in Lugol solution and Sudan IV respectively.

Under these conditions the neutral red droplets, the ellipsoids, the starch granules, and the fat globules could be distinctly seen and clearly differentiated in all stages of digestion, but the time required for the different processes in digestion could not be ascertained.

2. Amoebae were starved until they had no food vacuoles. Then they were put into a solution containing chilomonads which were stained with neutral red and left until they had eaten one or more specimens, after which they were mounted as described above and studied under low and high power continuously until the chilomonads were digested.

This method yielded results which have a definite bearing on the time required for the disappearance from the food vacuoles of the various substances in them and the processes involved.

3. Numerous amoebae were washed in solution similar to that in which they were raised, until the solution contained no chilomonads. They then were left in the solution 24 hours, i.e., until the food vacuoles they contained had practically disappeared, after which numerous chilomonads were added. The solution was now set aside and left half an hour; then the amoebae were again washed as described above, set aside and left half an hour, after which a number of amoebae were removed, fixed in Sudan IV and mounted under a cover glass supported with ridges of vaseline. This was repeated at various intervals up to five hours. The preparations were now examined under an oil immersion objective. This method is particularly well adapted to establish the times required for digestion of the different substances in *Chilomonas*.

The results obtained by the three methods are presented in the following paragraphs and in Figure 2 and Table I.

Shortly after the chilomonas in the food vacuole is dead it begins to round and the neutral red droplets begin to come to the surface where some of them fuse and form masses of considerable size. After 45 minutes nearly all of these droplets have reached the surface and some have passed beyond out into the vacuole (Fig. 2 *B*). During

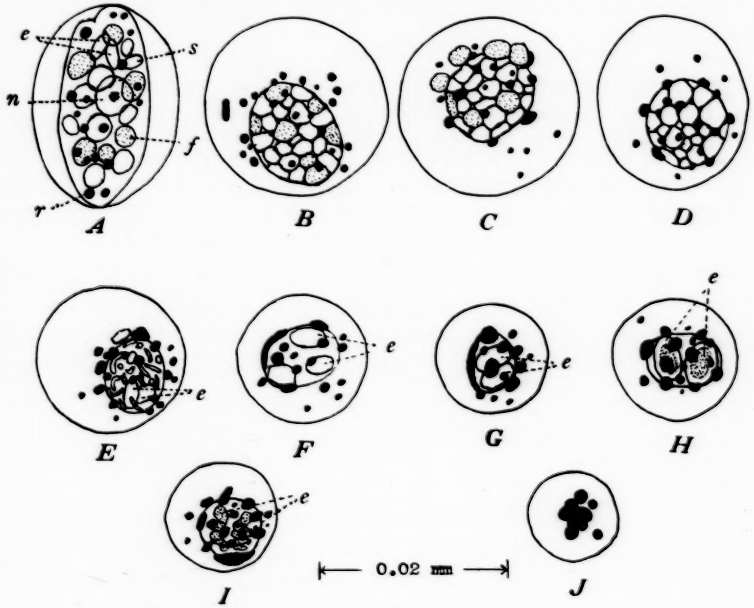

FIG. 2.—Camera outlines representing different stages in digestion of *Chilomonas* in *Amoeba proteus*. *A*, food vacuole immediately after death of the chilomonad in it; *e*, ellipsoids; *s*, starch grains; *f*, fat globules; *r*, neutral red droplets; *n*, nucleus; *mm*, projected scale; *B*, food vacuole 30 to 60 minutes after death of the chilomonad in it, neutral red droplets at the surface of the chilomonad; *C*, 60 to 90 minutes, fat globules at surface; *D*, 2 to 3 hours, no fat in the vacuole; *E*, 3 to 4 hours, starch grains partially disintegrated; *F*, starch grains disintegrated, a little starch paste left; *G*, 5 to 8 hours, nothing except ellipsoids and neutral red droplets left; *H*, central part of ellipsoids granular or vaculated; *I*, ellipsoids disintegrating; *J*, ellipsoids gone, neutral red droplets left.

this time the fat also begins to come to the surface, and 75 minutes after the chilomonad is dead nearly all has passed out but is still in contact with the surface (Fig. 2 *C*). Sometime after this the fat

disappears. This occurs between two and three hours after the chilomonad is dead (Fig. 2 *D*). The fat globules probably pass from the chilomonads out into the vacuole and then into the cytoplasm of the amoeba without undergoing any chemical or physical changes. At this time the ellipsoids, the starch grains, and the rest of the cytoplasm still appear to be intact and the neutral red droplets are

TABLE I

DIGESTION OF STARCH AND FAT IN *Amoeba proteus* FEEDING ON *Chilomonas*

Note that the fat globules come to the surface of the chilomonads in the food vacuoles between sixty and ninety minutes after they are eaten and leave the vacuole about an hour later and that it takes between four and five hours to digest the starch. There is probably no chemical change in the fat.

Time in Hours after Feeding	Number of Amoebae		Number and Condition of Chilomonads in Amoebae				
			Starch in Grains			Starch Paste	Total
	Containing Chilomonads	Containing No Chilomonads	Chilomonas Nearly Normal	Fat on Surface of Chilomonas	No Fat	No Fat	
0.5.....	3	2	17	0	0	2*	19
1.0.....	4	0	10	11?	1	0	22
1.5.....	9	10	7	26	8	0	41
2.0.....	9	3	3	17	20	0	40
3.0.....	11	5	1	0	15	0	16
4.0.....	10	1	0	0	9	4	13
5.0.....	8	1	2†	0	2	26	30

* These two vacuoles were very small; they were in the amoebae when the feeding period began and were much older than thirty minutes.

† These two vacuoles were large; they were the result of incomplete elimination of food, and consequently had been in the amoebae only a short time.

still in the vacuole, but outside of the chilomonad. The starch grains now begin to decrease in size and the proteins in the nucleus and the cytoplasm probably begin to hydrolyze and the entire remnant of the chilomonad rapidly decreases in size. Three to four hours after death, the starch grains are small and irregular in shape and the reddish color of the rest of the substances in specimens stained with neutral red has disappeared (Fig. 2 *E*). Four to five hours after death there is left in the chilomonads nothing but a

finely granular mass, two ellipsoids and a number of neutral red droplets. The granular mass surrounds the ellipsoids and the neutral red droplets are at the surface of it (Fig. 2 *F*). This mass becomes faintly blue with iodine, indicating that it still contains some starch. It soon disappears, however, leaving nothing but the ellipsoids surrounded by the neutral red droplets (Fig. 2 *G*). The ellipsoids now swell somewhat and the central portion becomes distinctly granular or vacuolated (Fig. 2 *H*). It can be clearly seen that this portion is more deeply stained than the outer layer. The ellipsoids probably contain fatty acid (Mast and Doyle, 1934*a*).[3] Sometime after this the surface of the ellipsoids becomes wrinkled or etched (Fig. 2 *I*) after which they soon disintegrate, leaving nothing visible in the vacuole but an aggregate of neutral red droplets (Fig. 2 *J*). The time required for the entire process is eight to twelve hours but this varies greatly in different individuals. The results presented above were obtained at 25° to 27° C.

What becomes of the neutral red droplets was not directly ascertained, but the following facts throw some light on this problem: (1) Neutral red droplets without vacuoles are frequently found scattered individually through the cytoplasm of amoebae. (2) The neutral red droplets stain the same with vital dyes and they have the same solubility in water, alcohol, acids and bases as the spherical bodies in amoebae. (3) The spherical bodies in amoebae are larger and much more numerous in specimens fed exclusively on *Chilomonas* which contains numerous neutral red granules than they are in specimens fed exclusively on *Colpidium* which contains relatively few.

These facts indicate that the neutral red droplets are not digested, and that after digestion of other substance is complete the food vacuoles disintegrate, releasing the neutral red droplets, which then become distributed through the cytoplasm and later function in the formation of the spherical bodies (Mast, 1926).

Crystals of different sizes are occasionally found in the food

[3] If a chilomonad is crushed by pressure on the cover-glass so as to force the ellipsoids out into the water they immediately disintegrate. This indicates that some of the substances in them are highly soluble in water. This lends support to the contention of Mast and Doyle (1934*a*) that the ellipsoids contain fatty acid.

vacuoles. We have found them only in vacuoles in which digestion is nearly complete. This indicates that the crystals are formed in the food vacuoles. The fact, however, that they occur so rarely in the vacuoles (probably in less than one in a hundred) greatly weakens the evidence in favor of this view. But if those found in the vacuoles are not formed there how did they get into the vacuoles? We have no answer.

Monas punctum

Two specimens of *Monas punctum* in one food vacuole in an amoeba were studied from time to time for 59 hours. The amoeba was then crushed. Both monads appeared to be dead but they looked precisely like living specimens, both in form and structure.

Food vacuoles each containing numerous monads were repeatedly found in amoebae especially in cultures which were rich in monads and poor in *Chilomonas* and *Colpidium*. An amoeba which contained a very large monad vacuole was crushed and the monads which had been in the vacuole, were examined and counted. There was no perceptible indication of digestion in any of them and many became active after they had been liberated. This vacuole, as previously stated, contained more than 1,200 monads (Fig. 1). In these cultures there were, scattered over the bottom, numerous masses which consisted of monads closely packed together. The monads were dead but they appeared normal in other respects. These masses were doubtless vacuoles which had been thrown off by the amoebae.

In a few amoebae there were found vacuoles containing monads which were distinctly yellowish in color and apparently partially digested.

These facts indicate that if digestion of *Monas punctum* actually occurs in *Amoeba proteus* it is unusual.

There are also a number of other organisms which are eaten and not digested, e.g. *Gonium*, various algae, and some mold spores.

ELIMINATION OF SOLID SUBSTANCE

Food in various stages of digestion and indigestible residue in the food vacuoles are eliminated in solid form. The process of elimina-

tion of these substances was seen in detail on several occasions. It occurs as follows:

The food vacuole containing the particles to be eliminated comes in contact with the plasmalemma at or near the posterior end of the amoeba. Here it remains a few moments (30± seconds) then the particles in the vacuole suddenly spurt out, going a considerable distance from the surface of the amoeba before they come to rest. This indicates that the whole system is under tension, that the contact of the vacuole with the plasmalemma causes it to break, and that contraction of the plasmagel and the plasmalemma forces the particles out through the opening.

After the particles have been discharged there often is a definite flow of plasmasol toward the break in the plasmalemma. This results in the formation of a pseudopod, but the pseudopod thus formed rarely becomes large and is always soon retracted. The formation of the pseudopod is doubtless due to local dissolution of the plasmagel by the action of the food vacuole. What the nature of this action is is not known, and it is also not known what processes are involved in the action of the food vacuole on the plasmalemma.

CHANGES IN AMOEBA DURING STARVATION

Specimens of *Amoeba proteus* were at various times isolated in culture fluid without food and studied from time to time in reference to size and structure. They were kept in moderate illumination and at room temperature which varied but little from 21°.

The length of life of the specimens isolated varied greatly. Some lived only a few days, while a few lived twenty-one days. During starvation *Amoeba* decreases in size, rapidly at first and then more slowly, until its volume has decreased about 95 per cent after which it disintegrates. The nucleus also decreases greatly in size but much less rapidly than the cytoplasm (Fig. 3 *A* and *B*); i.e., as the volume of the cell decreases the nucleo/cytoplasmic ratio increases. Chalkley (1931) made a more comprehensive study of this relation in amoebae which were increasing in size and found the same relation, i.e. he found that the nucleo/cytoplasmic ratio varies inversely with the volume of the cell.

During the decrease in volume the food vacuoles, the fat globules

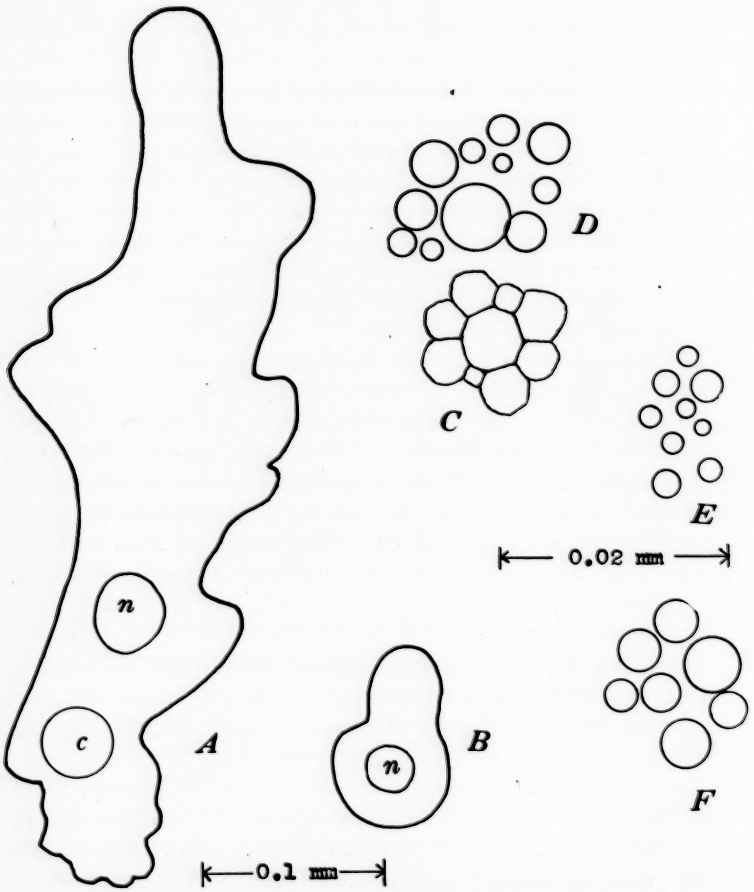

FIG. 3.—Camera outlines illustrating changes in *Amoeba proteus* during starvation. *A*, normal specimen; *B*, specimen after having been 15 days without food; *n*, nucleus; *C*, contractile vacuole; *mm*, projected scale; *C*, group of spherical bodies which had aggregated at the bottom of the plasmagel in *B; D*, spherical bodies from *B* after they had been separated. (Note that the angular surfaces became uniformly curved after the bodies were separated, indicating that they are plastic.) *E*, group of smallest spherical bodies found in amoebae which had been fed exclusively on *Colpidium* for 18 days; *F*, group of largest spherical bodies found in amoebae which had been fed nearly exclusively on *Chilomonas* for 25 days; *mm*, projected scale.

and the beta granules disappear, but the crystals, the spherical bodies and the alpha granules do not decrease greatly if at all, either in number or in size. They appear to become more abundant as starvation continues but this is doubtless due to the great decrease in other constituents of the cytoplasm. As a matter of fact the cytoplasm just before death contains only a trace of substance aside from these structures, all of which so far as is known have no immediate vital function.

As starvation proceeds movement decreases, fewer and shorter pseudopods are formed until the organism becomes nearly spherical and movement practically ceases. Shortly after this the organism usually disintegrates. During these final stages the cytoplasm becomes more and more fluid, the spherical bodies aggregate and settle first to the bottom of the plasmasol and then to the bottom of the plasmagel. The crystals collect in a fairly definite layer above the spherical bodies, and the nucleus comes to rest immediately above this, extending to a little above the center of the organism. The upper portion of the organism now consists of very finely granular substance. The spherical bodies clearly constitute the heaviest substance in *Amoeba*.

After the spherical bodies have aggregated, the surfaces in contact with each other are flat and the outlines of the bodies are angular, but if the bodies are now separated they become spherical again (Fig. 3 *C* and *D*). If the cover-glass is gently pressed down so as to apply continuous pressure to the spherical bodies after they have been forced out of the amoeba they assume various forms depending upon the nature and the position of particles in contact with them. If the pressure is now released they slowly become spherical again. This shows that the spherical bodies are definitely plastic but that they have a high viscosity. Immediately before the organism disintegrates, the crystals usually dissolve and the fluidity of the cytoplasm increases greatly. These facts indicate that an excessive amount of water has entered. In *Amoeba* death due to starvation is consequently associated with the destruction of the mechanism which normally regulates the water content.

THE RELATION BETWEEN THE KIND OF FOOD AND THE STRUCTURE OF AMOEBA PROTEUS

Some amoebae were put into a given amount of culture solution and fed exclusively on *Colpidium* for 18 days. Others were put into the same amount of culture solution of the same kind and fed nearly exclusively on *Chilomonas* for 24 days. The amoebae multiplied in both cultures and they appeared to be in excellent condition in both at the close of the feeding periods, which ended at the same time, the latter having been started seven days earlier than the former.

At this time groups of specimens were taken from both cultures and treated respectively with neutral red, Lugol solution and Sudan III. These specimens and also normal unstained specimens from both cultures were carefully examined under an oil immersion objective.

No difference in the structure of the cytoplasm in the amoebae from the two cultures was observed. But the chilomonas-fed specimens contained definitely more crystals and more spherical bodies than the colipidium-fed specimens. No difference in the size of the crystals was observed but the spherical bodies were markedly larger in the former (Fig. 3 *E* and *F*). This is of considerable importance for, as stated above, it indicates that the neutral red staining droplets found in abundance in *Chilomonas* but not in *Colpidium* function in the formation of the spherical bodies in *Amoeba*.

SUMMARY

1. *Amoeba proteus* feeds largely on *Chilomonas* and *Colpidium*. All food is ingested by means of food cups. As many as fourteen organisms are sometimes ingested in one cup.

2. Feeding occurs only in specimens which are attached to the substratum. Anything which facilitates attachment facilitates feeding.

3. *Amoeba proteus* will under certain conditions ingest any animal or plant that is not too large or too active, but it does not ingest them indiscriminately, e.g. it rarely takes *Monas* if *Chilomonas* or *Colpidium* is available.

4. *Chilomonas* after it is ingested, remains alive 3 to 18 minutes, *Colpidium* somewhat longer, and *Monas* more than 3.5 hours.

5. Vacuoles that contain more than one organism usually divide into as many as there are organisms. Vacuoles that contain one organism sometimes divide into two or more. If vacuoles contain indigestible organisms, e.g. *Monas*, they sometimes fuse and form giant vacuoles, containing as many as 1,250 individuals.

6. An active amoeba at room temperature consumes 50 to 100 chilomonads a day.

7. Some organisms which are eaten are not digested, e.g. *Monas*, *Gonium*, various algae, and some mould spores.

8. *Chilomonas* at room temperature is digested in 12 to 24 hours. The starch, the proteins and the ellipsoids are digested. The fats and the droplets which stain red with neutral red are not, but they become distributed in the cytoplasm. The digested substances probably pass out of the vacuoles by diffusion. How the fats get out of the vacuoles is not known. The neutral red droplets are released by disintegration of the vacuoles.

9. Vacuoles containing substances in various stages of digestion or indigestible substance, are often eliminated. These vacuoles break through the plasmagel and the plasmalemma owing to their liquefying action at the point of contact.

10. *Amoeba proteus* at room temperature can live without food 20 days or longer. It decreases in size about 95 per cent. The fat and the beta granules disappear, but the crystals, the spherical bodies, and the alpha granules do not disappear, and they probably do not decrease either in number or in size. Immediately before death the viscosity of the cytoplasm becomes nearly as low as that of water.

11. The structure of the cytoplasm in *Amoeba proteus* is not to any appreciable extent dependent upon the kind of food eaten, but the content of it is.

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THE EFFECT OF CERTAIN SALTS OF SEA WATER UPON REPRODUCTION IN THE MARINE AMOEBA, FLABELLULA MIRA SCHAEFFER[1]

(Eight figures)

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WITH the exception of the investigations of Pantin (1923, 1924, 1926*a*, 1926*b*, 1930*a*, 1930*b*, 1930*c*, 1931), in which he has made a rather complete study of certain "limax marine amoebae," and of Hopkins (1929*a*, 1930, 1931) very little experimentation of a physiological nature has been carried out upon marine amoebae. Hopkins (1930) stated that *Flabellula mira* Schaeffer, a marine amoeba, could be cultured in "sea-water diluted many times to sea-water concentrated six or eight times." This indicated that such a species might be an excellent form upon which to study the effects of salts of sea water and dilutions of sea water, and might permit an interesting comparison of fresh-water and marine amoebae.

If certain fundamental facts could be established for a marine amoeba which is described and identified and has a wide range of osmotic tolerance, a new field of investigation would be open for other problems in cell physiology.

The present paper gives the results of a statistical study of *Flabellula mira* Schaeffer in which reproduction was used as a criterion of the physiological state of an amoeba.

MATERIAL AND METHODS

The amoebae used in the experiments were from a single clone from a single amoeba isolated in September, 1933, from amoebae collected in tidal pools at Tortugas by D. L. Hopkins in 1930 and identified by A. A. Schaeffer as *Flabellula mira* Schaeffer.

All the salts were C. P., Baker's Analyzed. The impurities in the

[1] The author wishes to express her appreciation to Professor D. L. Hopkins, under whose direction the work was carried out, for his kindly help and criticism.

salts were undoubtedly less than the unknown salts in the wheat used as the source of food. The distilled water, which was tested and found to be of high purity, was run directly from a Barnstead still into a Pyrex carboy. The H-ion concentration was determined colorimetrically by the method described by Brown (1924) with the apparatus Model 5B made by the La Motte Chemical Products Company.

The artificial sea water in which the stock cultures were grown and which was used in the experiments is that described by McClendon (1916), who gives the molecular concentration of each salt which is isotonic with sea water, and the quantity of each stock solution used in making artificial sea water.

The stock amoebae were cultured at 30° C. in 6 cm. Petri dishes in approximately 20 cc. of artificial sea water containing four grains of wheat. The food of the amoebae consisted of bacteria which lived on the wheat and were carried from one culture to another with the inoculation of amoebae. The Petri dishes, the artificial sea water, the wheat, and the pipettes used in making the inoculations were sterilized. Although no study was made of the bacteria in the cultures, the technique which was employed kept the cultures free from all protozoa other than *Flabellula mira* and assured fairly uniform conditions of food for the amoebae.

All the glassware used in the experiments was washed in soap and water, then in potassium bichromate–sulphuric acid cleaning solution, and rinsed at least six times in distilled water.

The experimental cultures were set up as follows:

Fifty cubic centimeters of medium was placed in each of four 125 cc. Pyrex flasks, together with ten grains of wheat. Each flask was plugged with cotton, and the whole series of flasks for each experiment was sterilized in the autoclave at 15 lb. pressure for 30 minutes.

When the flasks with the solutions were sufficiently cool, 0.2 cc. of fluid containing *Flabellula mira* from the stock culture was added to each flask. The flasks were then placed in an incubator at 30° C. for 7 days. On the seventh day ten counts were made of the amoebae in each flask. The figures for each point on the curves are based on forty counts, ten for each of the four solutions of the same concentra-

tion. Thirty-two solutions, four at each concentration, constituted an experiment.

Flabellula mira reproduced under favorable conditions at the rate of approximately 1.5 divisions a day, so that a culture on the seventh day after inoculation was very populous. The diameter of *F. mira* when rounded up was from 15 μ to 20 μ. Consequently, it was not possible to count the amoebae one by one under a microscope by removing each amoebae with a pipette, as Pace (1933) did in working on reproduction in *Amoeba proteus*.

The amoebae were counted as follows: The culture was thoroughly stirred with a sterile pipette. A drop of culture fluid was placed on a Levy counting-chamber under a microscope, and the number of amoebae in the nine divisions of the chamber were counted. The amoebae were counted in ten samples from each culture in every case. The results are based upon over seven thousand individual counts.

In order to determine the accuracy of the method used, fifty counts were made on one culture. The standard deviation of the average, or the standard error, was found to be ± 0.70, or a probable error of ± 0.47. These fifty counts were averaged in groups of ten. The standard error of these averages was ± 0.56, or a probable error of ± 0.37 The probable error is not given for the results of each individual experiment, but these calculations show that the error in the method is not great. The numerical results for different series cannot be exactly compared, since the initial number of amoebae was not always constant; but the initial number probably does not determine the final number.

When an inoculation was made, the amoebae in the stock cultures were counted as described. The number in 1 cc. could be calculated by multiplying the average of the ten counts for 0.9 cu. mm. by 1,111. Since it was desirable to inoculate a constant amount of fluid, so that the salt concentration of the inoculum might be constant, rather than a constant number of amoebae, 0.2 cc. of fluid was inoculated into each flask, and the number of amoebae in 0.2 cc. calculated.

The H-ion concentration of each solution was determined at the beginning of the experiments and of the solution in each flask at the end of the experiments, with the exception of a few of the first series.

EXPERIMENTS AND RESULTS

I. The Relation between Salts and Their Concentration and Reproduction in Flabellula mira

The investigation of the relation of concentrations of different combinations of the common salts in sea water upon reproduction in

TABLE I

THE NUMBER OF CUBIC CENTIMETERS OF ISOTONIC SOLUTION OF EACH SALT WHICH WERE COMBINED TO GIVE A LITER OF SOLUTION

Combination	Salts Present	Isotonic Solution (Cc.)	Combination	Salts Present	Isotonic Solution (Cc.)
1A	$CaCl_2$	32.9	6	$MgCl_2$	72.4
	NaCl	967.1		KCl	18.8
				NaCl	908.8
1B	$CaCl_2$	250.0			
	NaCl	750.0	7	$CaCl_2$	32.2
				KCl	19.7
2	$MgCl_2$	94.5		NaCl	948.1
	NaCl	925.5			
			8	$CaCl_2$	261.7
3	$CaCl_2$	620.9		$MgCl_2$	583.9
	KCl	379.1		KCl	154.4
4	$MgCl_2$	793.3	9	$CaCl_2$	30.0
	KCl	206.7		$MgCl_2$	70.2
				KCl	18.3
5A	$CaCl_2$	30.5		NaCl	881.5
	$MgCl_2$	71.7			
	NaCl	897.8	10	$CaCl_2$	29.0 0.38 M
			(artificial	$MgCl_2$	67.9 0.37 M
5B	$CaCl_2$	29.0	sea water*)	$MgSO_4$	29.5 0.975 M
	$MgCl_2$	145.6		KCl	17.7 0.577 M
	NaCl	852.0		NaCl	852.0 0.568 M
				NaBr	1.4 0.565 M
		(1026.0 total volume)		$NaHCO_3$	2.5 0.93 M

* The artificial sea water is that given by McClendon (1916).

Flabellula mira may be divided into five groups of experiments. Each group will be discussed separately. Unless there is a specific reference to the ratio of one salt to another, the ratio of the salts in the mixtures was that which occurs in sea water. The solutions in which the concentration of salt was above that of sea water were called "concentrations," while the solutions in which the salt concentration was below sea water were called "dilutions." The so-

lution which was isotonic with sea water was called 100 per cent. A solution containing 90 cc. isotonic mixture to 10 cc. distilled water was called a dilution of 90, or 90 dilution. Table I gives the number of cubic centimeters of isotonic solution of each salt which were combined to give a liter of solution in making the various combinations used.

1. *Reproduction in concentrations and dilutions of artificial sea water.*—The results of three sets of experiments on reproduction of

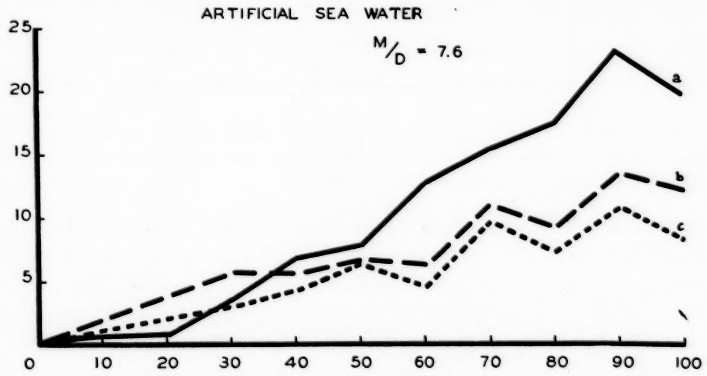

FIG. 1.—The relation of reproduction in *Flabellula mira* to dilutions of artificial sea water. Abscissae equal artificial sea water in percentages. Ordinates equal number of amoebae per 1 cu. mm. *M/D* equals monovalent/divalent-cation ratio.

Flabellula mira in dilutions of sea water (curves *a*, *b*, and *c*) are shown in Figure 1, and the results of one series of experiments in concentrations of sea water are shown in Figure 2, with the number of amoebae per 1 cu. mm. plotted as ordinates and the dilutions and concentrations in terms of 100 per cent isotonic artificial sea water as abscissae. Although the actual number of amoebae present in the first experiments was higher in the higher concentrations than in the second and third experiments, the general trend of the three curves is the same. The optimum for each is definitely at 90 artificial sea water.

There was no reproduction in distilled water.

2. *Reproduction in isotonic solutions of single salts.*—In order to check the negative results obtained from the first series of experi-

ments with isotonic solutions of single salts, cultures were set up in Petri dishes. These cultures could be carefully examined directly

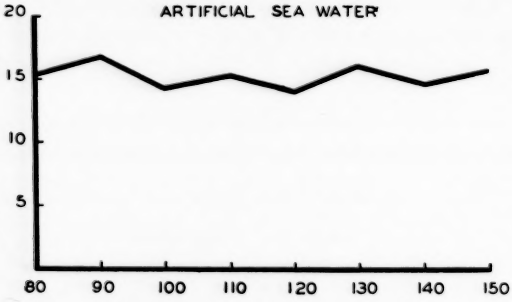

FIG. 2.—The relation of reproduction in *Flabellula mira* to dilutions and concentrations of artificial sea water. Abscissae equal artificial sea water in percentages. Ordinates equal number of amoebae per 1 cu. mm.

under the microscope. No amoebae were found in the solutions of single salts, but the control showed a high rate of reproduction.

3. *Reproduction in isotonic mixtures of two salts and dilutions.*—*$CaCl_2$+NaCl.*—Two types of experiments were carried out with

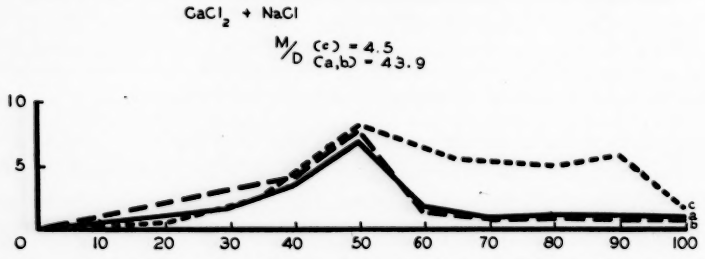

FIG. 3.—The relation of reproduction in *Flabellula mira* to dilutions of isotonic mixtures of $CaCl_2$+NaCl. Curves *a* and *b* represent mixture 1A with ratio as in sea water. Curve *c* represents mixture 1B (25 cc. isotonic $CaCl_2$ to 75 cc. isotonic NaCl). Abscissae equal solution in percentages. Ordinates equal number of amoebae per 1 cu. mm. *M/D* equals monovalent/divalent-cation ratio.

the mixture of $CaCl_2$ and NaCl. The results of two sets of experiments on reproduction in a series of dilutions of a mixture of $CaCl_2$ and NaCl with the ratio of NaCl/$CaCl_2$ the same as in sea water (combination 1 A, Table I) are shown in Figure 3, *a* and *b*. The curves show

a definite optimum for reproduction in the 50 dilution. The second series gave results almost identical with those of the first series.

However, when the molecular ratio of $NaCl/CaCl_2$ was changed from 43.9, as it exists in sea water, to 4.5, which is the molecular ratio of a solution containing 75 cc. isotonic NaCl solution to 25 cc. isotonic $CaCl_2$ solution (combination 1B, Table I), the shape of the dilution curve, shown as *c* in Figure 3 was considerably changed. Although the optimum was still at 50 dilution, the dilutions from 50 to 90 gave practically the same rate of reproduction. The results show definitely that good reproduction is possible in a solution in which the proportion of calcium to sodium is very much greater than in sea water.

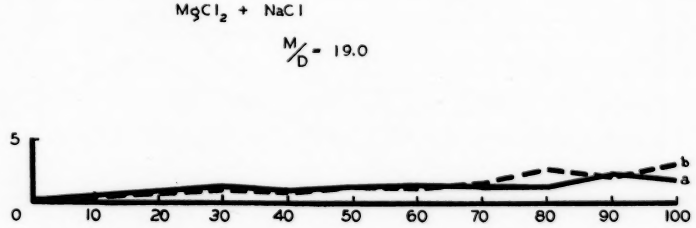

FIG. 4.—The relation of reproduction in *Flabellula mira* to dilutions of an isotonic mixture of $MgCl_2$+NaCl. Abscissae equal solution in percentages. Ordinates equal number of amoebae per 1 cu. mm. *M/D* equals monovalent/divalent-cation ratio.

$MgCl_2$+NaCl.—The results of two sets of experiments on reproduction in a series of dilutions of a mixture of $MgCl_2$ and NaCl (combination 2, Table I) are shown in Figure 4. Although there is no definite optimum, there was some reproduction at all dilutions from 20 to 100. The amoebae which were counted appeared to be normal individuals.

$CaCl_2$+KCl and $MgCl_2$+KCl.—The results of the experiments using a series of dilutions of a mixture of $CaCl_2$ and KCl (combination 3, Table I) and of $MgCl_2$ and KCl (combination 4, Table I) were so definitely negative that a second check experiment was not carried out with either mixture. There was no reproduction at any dilution.

4. *Reproduction in isotonic mixtures of three salts and dilutions.*—There were two types of series of experiments carried out with the

combination of $CaCl_2$, $MgCl_2$, and NaCl; one (combination 5A, Table I) in which the ratios of the salts were to each other as the salts are to each other in sea water, the other (combination 5B, Table I) in which the ratio was such that the magnesium was equal to the total magnesium in sea water, not to the $MgCl_2$ alone. In the latter experiments the proportion of $MgCl_2$ was much larger than in the former.

Figure 5 shows two curves (*a* and *b*) for reproduction in the first type of solution and one curve (*c*) for reproduction in the second

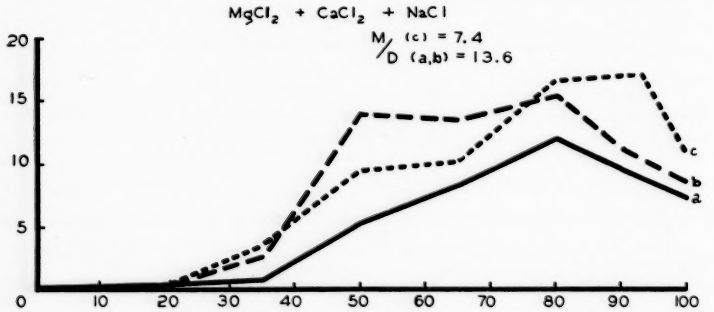

FIG. 5.—The relation of reproduction in *Flabellula mira* to dilutions of isotonic mixtures of $CaCl_2+MgCl_2+NaCl$. Curves *a* and *b* represent mixtures 5A with ratio as in sea water. Curve *c* represents mixtures 5B (Magnesium equal to total magnesium in sea water). Abscissae equal solution in percentages. Ordinates equal number of amoebae per 1 cu. mm. *M*/*D* equals monovalent/divalent-cation ratio.

type. Although curve *b* departs from *a* considerably at 50, both curves show an optimum at 80. While curve *c* follows *a* in general, it shows a definite optimum at 90.

$MgCl_2+KCl+NaCl$ and $CaCl_2+MgCl_2+KCl$.—The combination of $MgCl_2+KCl+NaCl$ (combination 6, Table I) and of $CaCl_2+MgCl_2+KCl$ (combination 8, Table I) produced solutions in which there was no reproduction at 100 or at any dilution.

$CaCl_2+KCl+NaCl$.—The solution resulting from a combination of $CaCl_2+KCl+NaCl$ (combination 7, Table I) permitted reproduction from 20 to 100 dilutions, as shown in Figure 6. The two curves representing two identical series of experiments carried out at different times follow each other quite closely in their general shape, with the optimum at 65. The addition of KCl to a solution of $CaCl_2+$

NaCl raises the optimum for reproduction from 50, as shown in Figure 3, to 65, as shown in Figure 6.

5. *Reproduction in isotonic mixtures of four salts and dilutions: $CaCl_2+MgCl_2+KCl+NaCl$.*—The curves for the two series of experiments on reproduction in solutions of the four salts, $CaCl_2$, $MgCl_2$, KCl, and NaCl, combined (combination 9, Table I) as shown in Figure 7, are similar. These curves are very much like the first curve for reproduction in dilutions of artificial sea water in Figure 1 except that the optimum is at 80 in the combination of the

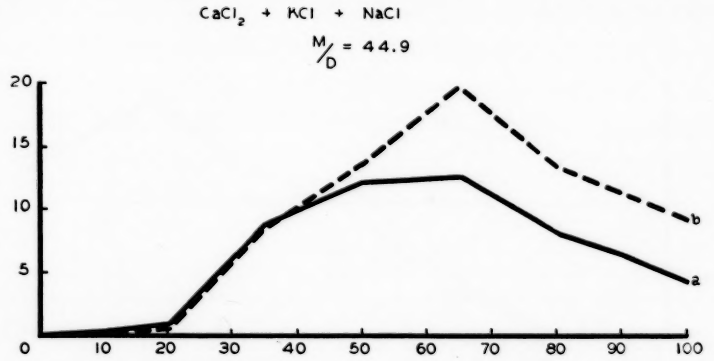

FIG. 6.—The relation of reproduction in *Flabellula mira* to dilutions of an isotonic mixture of $CaCl_2+KCl+NaCl$. Abscissae equal solution in percentages. Ordinates equal number of amoebae per 1 cu. mm. M/D equals monovalent/divalent-cation ratio.

four salts and at 90 in artificial sea water. If Figure 5 is compared with Figure 7, it can be seen that the addition of KCl to a solution of $CaCl_2+MgCl_2+NaCl$ has little effect upon the shape of the curve for reproduction in dilutions of the solution.

When comparison is made between Figure 6 and Figure 7, it appears evident that the addition of $MgCl_2$ to a solution of $CaCl_2+KCl+NaCl$ increases the optimum for reproduction from 65 dilution to 80.

II. Experiments in Which HCl Was Added to Artificial Sea Water to Vary the H-Ion Concentration

A series of experiments in which the H-ion concentration was varied, carried out as carefully and thoroughly as any other phase of

the work reported here in detail, showed that within the ranges of pH values in the experiments the H-ion concentration of the solutions did not affect the results of these experiments.

III. Experiments in Which the Ratio of NaCl to $CaCl_2$ Was Varied but the Osmotic Value Was Constant

Three sets of experiments were carried out in which the combination of isotonic solutions of NaCl and $CaCl_2$ was varied. One hundred per cent represented an isotonic solution of NaCl. Ninety-five per cent represented 95 cc. isotonic solution of NaCl to 5 cc. isotonic solution of $CaCl_2$.

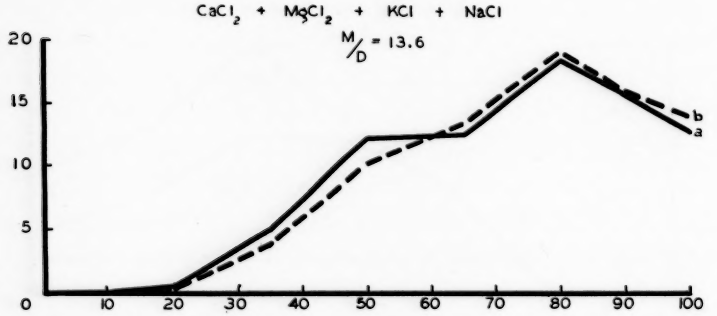

FIG. 7.—The relation of reproduction in *Flabellula mira* to dilutions of an isotonic mixture of $CaCl_2+MgCl_2+KCl+NaCl$. Abscissae equal solution in percentages. Ordinates equal number of amoebae per 1 cu. mm. M/D equals monovalent/divalent-cation ratio.

The average for these experiments is shown in Figure 8. The experiments show that there is a definite range of $NaCl/CaCl_2$ ratios which is favorable for reproduction and that beyond this range either too much NaCl or too much $CaCl_2$ is not favorable for reproduction. At 75, the optimum, the molecular ratio $NaCl/CaCl_2$ is 4.5.

IV. Reproduction as a Criterion of Activity

The relation of the initial number of amoebae inoculated into the cultures to the final number of amoebae is of interest. As described above, the number of amoebae inoculated could be calculated. Table II, based on counts in 100 per cent solutions, shows that the number of amoebae after 7 days is not directly proportional to the number of

amoebae inoculated. Experiments in which the experimental solutions were the same are grouped together. In Experiment O, for instance, the number of amoebae inoculated was 666 and the final number was 388,850; while in the initial count of Experiment T, 644 were present but the final number was 422,200. The greater initial number did not produce a greater final number. The fact that the final number of organisms at the optimum of a culture is not dependent upon the initial number of organisms inoculated is in harmony with the conclusions of Graham-Smith (1920) from his work with the bacterium *Staphylococcus aureus*.

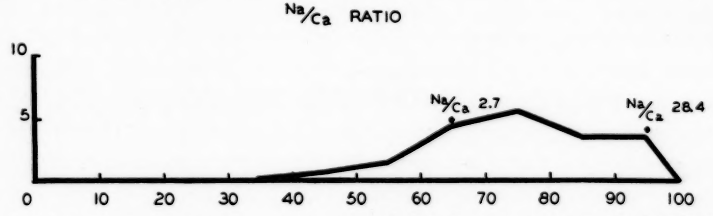

FIG. 8.—Average of three experiments on the relation of reproduction in *Flabellula mira* to isotonic solutions in which the ratio of NaCl to $CaCl_2$ is varied. Abscissae equal percentage of isotonic NaCl solution in the mixture of isotonic NaCl solution and isotonic $CaCl_2$ solution. Ordinates equal number of amoebae per 1 cu. mm.

Since the temperature was kept constant in the present experiments within 1° C. variation in either direction, the variation must have been due to variation in the rates of division of different amoebae or to variation in the bacterial growth. It is possible that the effect of the solutions upon the bacteria limit the reproduction in the amoebae, and thus the solutions do not directly affect the amoebae but determine their food supply. Although it was not possible to estimate the number of bacteria present without using special technique, it was noted that, with the exception of the cultures in the experiment on the effect of H-ion concentration in which the values were from pH 3.5 to pH 4.8, bacteria were present in abundance.

DISCUSSION

Reproduction in artificial sea water and in a mixture of four salts.—Pantin (1926*a*), in discussing the artificial sea water which he used in his work on his "limax amoebae," said the sulphate ion was found to

be unnecessary. A comparison of the results of the present experiments on reproduction in *Flabellula mira* in McClendon's artificial sea water and in a mixture of isotonic solution of $CaCl_2+MgCl_2+KCl+NaCl$ supports Pantin's conclusion. However, since 0.2 cc. of artificial sea water was added to each 50 cc. of experimental solution

TABLE II

THE INITIAL AND FINAL NUMBER OF AMOEBAE IN 50 CC. OF SOLUTION AND THE NUMBER OF AMOEBAE RESULTING FROM THE DIVISION OF ONE OF THE ORIGINAL AMOEBAE IN CERTAIN EXPERIMENTS

There is an increase in the number of amoebae with an increase in the number of salts present.

Solution	Experiment	Initial Number per 50 Cc.	Final Number per 50 Cc.	Number of Descendants of Each Initial Amoeba
Sea water	B	822	999,900	1,216
	C	889	722,150	812
	F	1,066	611,050	573
	H	1,711	388,850	221
$CaCl_2+NaCl$	D	1,111	50,000	45
	J	1,066	27,775	26
$MgCl_2+NaCl$	L	977	83,325	85
	M	755	144,430	191
$CaCl_2+MgCl_2+NaCl$	O	666	388,850	583
	T	644	422,200	656
$CaCl_2+KCl+NaCl$	R	711	222,200	312
	U	599	422,200	705
$CaCl_2+MgCl_2+KCl+NaCl$	V	755	666,600	882
	W	622	722,150	1,161

no attempt will be made to discuss the absolute deficiency of any ion which was present in artificial sea water.

The optimum in the experiments in artificial sea water was not at the osmotic concentration of sea water but at the 90 dilution, and reproduction was practically constant from 100 to 150 isotonic artificial sea water. Pantin (1923) observed the effects of change in osmotic pressure on his marine amoebae over short periods of time. That *Flabellula mira* lived and reproduced for many days under con-

ditions in which the osmotic pressure was varied considerably from the normal indicates not only that it can adjust itself but also that it can adapt itself to its environment to a great extent. The fact that some amoebae were present in the 10 and 20 dilutions shows that at least some of the amoebae adjusted themselves to those particular environments and reproduced. The chance of finding one of the original amoebae in the final samples, as calculated for a typical case, was found to be about 1 in 50.

Reproduction in three or fewer salts.—There was no reproduction in isotonic solutions of the single salts. This is in perfect harmony with the results of Hopkins (1930) in his work on *Flabellula mira*.

The results of the experiments on the mixtures of salts would indicate that both calcium and sodium are necessary for reproduction if it were not for the very definite, although meager, reproduction in $MgCl_2$+NaCl. It is definite, however, that calcium is necessary for a high rate of reproduction. It is just possible that in the absence of potassium in the experimental solution the amount of calcium in the inoculum was enough to be effective. This is not true for sodium, for in all the solutions in which this was not in the experimental mixture no reproduction occurred. When sodium was not present, the amount of potassium necessarily added to keep the 100 solution isotonic with sea water was probably toxic. Mast and Prosser (1932), in their work on *Amoeba proteus*, found that KCl was relatively much more toxic than NaCl, $CaCl_2$, or $MgCl_2$; and many other investigators have reported the same thing. The amount of KCl in my experiment might possibly not be enough to satisfy the need for monovalent cations.

From the present experiments it appears quite certain that calcium is necessary for a high rate of reproduction in *Flabellula mira*. Although Pantin (1926*b*) found movement in a mixture of $MgCl_2$+$CaCl_2$, he said that it gradually fell off and that only when all four cations of sea water were present was movement normal indefinitely. He also found movement in KCl+$CaCl_2$ but said that viability in this was less than in NaCl+$CaCl_2$ mixtures. Thus, although NaCl is not necessary for locomotion in Pantin's "limax amoebae," it is probable that it is necessary for continued growth and reproduction in it as well as in *Flabellula mira*.

In studying the effect of salts on reproduction, it is not possible to draw conclusions as to the specific effect upon any one part of the cell or any one mechanism. As to the actual action of the various cations, there is little general agreement. To what extent the effect of concentration of salts can be separated from the osmotic effect in the present experiments is a question. The antagonistic action of the cations is, however, very clear. When to $CaCl_2+KCl$, which gives negative results, NaCl is added, there is a high rate of reproduction. When to $MgCl_2+NaCl$, in which reproduction is only fair, $CaCl_2$ is added, reproduction is excellent. The solution $MgCl_2+KCl+NaCl$, which permits no reproduction, gives excellent reproduction when $CaCl_2$ is added; and again a solution of $CaCl_2+MgCl_2+KCl$ allows no reproduction unless NaCl is added.

Chambers and Reznikoff (1925) suggested that the phenomenon of antagonism depends upon sodium and calcium or potassium and calcium protein salts. Many investigators have shown that there is a definite antagonistic action upon the membrane of a cell. Hopkins (1929*b*) has suggested that the divalent cations tend to cause gelation while the monovalent cations tend to cause solation in *Amoeba proteus*. Consequently, only when monovalent and divalent cations are in a fairly definite ratio is the viscosity such that the cell can function adequately.

Monovalent/divalent salt ratio.—On each figure in the text the ratio of monovalent cations to divalent cations in each of the different solutions is recorded. It is interesting to observe that in the experiments in which the optimum reproduction is in fairly dilute solutions the monovalent/divalent-cation ratio is larger than in the experiments in which the optimum reproduction is in less dilute solutions. That is, the optimum monovalent/divalent-cation ratio varies roughly inversely with the optimum dilution; or the smaller the total concentration of salts in the solution, the larger must be the proportion of monovalent ions for optimum reproduction.

The experiments on the relation of the $NaCl/CaCl_2$ ratio show that reproduction is possible over a wide range of variation in the $NaCl/CaCl_2$ ratio from 43.9 to 2.7 or from the proportion in sea water to 65 $NaCl/35$ $CaCl_2$. Curve *c* in Figure 3 is a dilution curve of the mixture which was found to be the optimum in the average

of the three experiments on $NaCl/CaCl_2$ ratio. A high rate of reproduction occurs in a far greater range of dilutions when the $NaCl/CaCl_2$ ratio is 4.5 than when it is 43.9, as in sea water.

Pantin (1926*a*) reported a high rate of locomotion in solutions of $NaCl+CaCl_2$ in which the Na/Ca ratio varied from a little over 4 to somewhat over 64 with his "limax amoebae." The present experiments show that reproduction is possible in *Flabellula mira* in solutions of $NaCl+CaCl_2$ in which the Na/Ca ratio varies from 2.7 to 43.9. Pantin stated that the optimum Na/Ca ratio was lower than the ratio in sea water. The experiments on *F. mira* show that this is also true for reproduction in this form.

H-ion concentration.—Pace (1933), in studying reproduction in *Amoeba proteus*, concluded that the variation in H-ion concentration did not invalidate his results. The same thing can be said in regard to my experiments on reproduction in *Flabellula mira*.

Marine amoebae compared with fresh-water amoebae.—A comparison of Pace's investigation upon *Amoeba proteus* with the present study, which is similar at least in the method of approach, would indicate that there are great differences between the reactions of fresh-water amoebae and marine amoebae. Adolph (1925), after comparing fresh-water and marine organisms, concluded: "Thus it turns out that while the essential physiological constitution of the tissues of the two groups of animals does not differ, the limiting factors for the proper functioning of their regulatory integuments differ vitally." Perhaps "the essential physiological constitution" of the protoplasm of the two groups of animals also does differ.

SUMMARY

1. The final number of individuals of *Flabellula mira* in a culture is not closely correlated with the number of amoebae in the inoculum.

2. *Flabellula mira* is able to reproduce in concentrations of artificial sea water from 20 to at least 150. Reproduction below 20 is rare, and no reproduction occurs in distilled water.

3. Sodium and, quite probably, calcium are necessary for optimum reproduction in *Flabellula mira*. Reproduction occurs in solutions in which the Na/Ca ratio varies from 2.7 to 43.9.

4. Reproduction in a mixture of $CaCl_2+MgCl_2+KCl+NaCl$ in the proportion one to another in which they exist in artificial sea water is nearly comparable to that in artificial sea water.

5. No reproduction occurs in isotonic solutions of single salts or in the following mixtures: $CaCl_2+KCl$, $MgCl_2+KCl$, $MgCl_2+KCl+NaCl$, $CaCl_2+MgCl_2+KCl$.

6. A high rate of reproduction occurs in mixtures of $CaCl_2+MgCl_2+NaCl$ and $CaCl_2+KCl+NaCl$. Some reproduction occurs in mixtures of $CaCl_2+NaCl$ and $MgCl_2+NaCl$. There is probably a trace of $CaCl_2$ in the latter from the inoculum.

7. There is a correlation between the optimum dilution or osmotic concentration and the monovalent/divalent-cation ratio.

8. Within the ranges of pH values in the experiments the H-ion concentration of the solutions do not affect the results of these experiments.

9. Reproduction is greatest in artificial sea water and decreases with the removal of each salt.

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THE EFFECT OF TEMPERATURE ON THE DEVELOPMENT OF RANA PIPIENS[1]

(Eight figures)

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VARIOUS attempts have been made to study the effects of temperature on early development. Among the first quantitative studies in this field were those on the frog's egg by Lillie and Knowlton (1897) on *Rana* (virescens) *pipiens*, and by Hertwig (1898) on *R. fusca*. The former workers were pioneers in quantitative studies of this kind and were the first to present their data in the form of graphs. The studies of Hertwig were much more extensive, and led him to the conclusion that development undergoes a regular acceleration with increase of temperature, which is primarily due to chemical processes. Later workers continued such studies on other species. An extensive summary of such work may be found in Needham (1931).

The present work was undertaken to study the behavior of the developing egg of *Rana pipiens* to temperature, with the special purpose of comparing separate processes. The studies have been restricted to such phenomena as could be observed without histological preparations. They include appearance of first cleavage furrow, following cleavages, gastrulation, neural fold formation, and growth. An attempt has been made to study these processes over the range of temperature tolerated by this species. When several processes have occurred simultaneously, an attempt has been made to study each separately and to correlate the rates of one with the others.

MATERIAL AND TECHNIQUE

Frogs have been obtained from dealers in Vermont. Ovulation has been stimulated at all times of the year by the technique of pituitary

[1] In presenting this work, it is a pleasure to acknowledge the patient and constant aid of Dr. L. G. Barth, at whose suggestion it was begun.

injection, or in some cases by implantation. The method used has been that, essentially, summarized by Rugh (1934). The majority of experiments has been performed between October and May. Storage of animals has been accomplished up to 2 weeks, preparatory to experimental use, in running cold water, or in a refrigerator at 12° C. Eggs obtained have been fertilized, up to 99 per cent in some cases, and have developed normally. The material obtained by artificial stimulation of ovulation has not been different from that obtained under natural conditions. The principal difficulty encountered was the necessity of using eggs within 24 hours after ovulation had started, owing to deterioration of such eggs if left in the uterus for longer periods.

Constant temperature has been maintained by the use of ovens with thermostat devices. Lower than laboratory temperatures were obtained by use of such ovens kept in a cold room. Thermometer readings are accurate to ±0.1° C. For any experiment, temperature measurements were made at the beginning of the experiment and during the course of the experiment whenever observations were made of the experimental material. Experiments showing fluctuations greater than 1° during the entire course of the experiment have been discarded.

The medium used for fertilization and development has been spring water. Prior to the beginning of an experiment, the finger bowls containing spring water for such experiments were brought to the temperature desired. Care was taken to prevent evaporation. The size of dish used varied in different experiments, but the density of eggs per dish was approximately twenty-five eggs per 100 cc. of water, with a depth of about 5 cm. of water.

In experiments which have been timed from fertilization the general procedure has been as follows: A sperm suspension has been prepared by macerating the testes of a frog in 5 cc. of spring water. This has been permitted to stand from 15 minutes to 2 hours. When needed for fertilization, this suspension has been spread in a thin layer over a small Stender dish, or finger bowl, and the eggs of an ovulating female have been squeezed out into it. The eggs have been permitted to remain in the undiluted sperm suspension for 8 minutes (to permit sufficient sperm penetration of the jelly). Then they have

been placed in water at the experimental temperatures, where the larger masses were cut up and separated to permit a freer surface for respiration. Simultaneous experiments were run at from two to eight temperatures. Deviations in general technique will be brought out during the discussion of separate experiments.

RESULTS

1. EFFECT OF TEMPERATURE ON FIRST CLEAVAGE

After fertilization the first end-point, which is definite enough to study from a quantitative point of view, is the appearance of the first cleavage furrow. In time it is preceded by the rotation of the egg and the appearance of the gray crescent. No attempt has been made to study the time relations of these, since they are much more variable at the same temperature for eggs of a single batch. At room temperature (21° C.) rotation has been observed in different batches of eggs from 25 to 45 minutes after fertilization. Furthermore, at temperatures below 5° C., the eggs may not rotate at all, in many cases; but cleavage may occur notwithstanding this circumstance.

The time of appearance of the first cleavage furrow varies with temperature and can be localized at temperatures above 9° within 2 minutes after its appearance. At lower temperatures it can be determined within 5 minutes. In normal batches of eggs at temperatures above 10°, the interval between the time the first egg cleaves and the time for 50 per cent to cleave is from 5 to 10 minutes. At lower temperatures the variation is greater and may be as much as 1 hour at 5°. Eggs placed at a constant temperature shortly after fertilization can cleave between 4° and 32°.

In Figure 1 are plotted the composite results for the effect of temperature on the time necessary for the appearance of the first cleavage. Abscissae represent temperatures in degrees centigrade; ordinates represent times from fertilization to the appearance of the cleavage furrow. Each point represents the time necessary for 50 per cent of a batch to attain the desired stage. Data from at least twenty-five different females are represented. A curve has been drawn through the points for use in interpolation.

An acceleration in first cleavage due to temperature has been noted up to 31°, beyond which there is abnormal cleavage; and at 32°

very few eggs show a normal first cleavage furrow. It is to be noted, in considering the variations among these results, that the tolerance of different batches of eggs varies. Tolerance also depends upon the time after fertilization when the eggs are placed at any particular

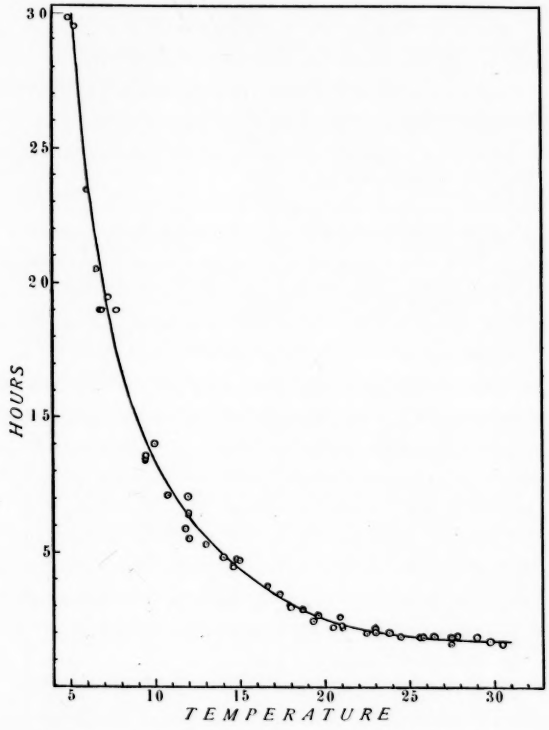

FIG. 1.—Temperature-time relation for the beginning of first cleavage. Each point represents an average of data from eggs of one female.

temperature. Thus in an experiment at 3.8° only eggs placed at this temperature later than an hour after fertilization cleaved. In those placed earlier, merely an aster-like structure appeared at the surface, but no actual furrow formed. Cleavage furrows which do occur outside the temperature range 5–32° are abnormal. They are, in many cases, through one side of the egg, so that the resulting blastomeres

Page 293: Fig. 1. On the ordinate axis, substitute for the numbers: 15, 20, 25, 30, the numbers below:
10, 15, 20, 25.

are unequal. The furrow is much wider than normal, and may not reach the vegetal pole. In many cases there may be multiple furrow formation, a second furrow starting at right angles to the first, shortly after that has appeared. This latter phenomenon may be an indication of polyspermy (cf. O. and R. Hertwig, 1887).

Below 3° the first cleavage is indefinitely inhibited, and eggs have been left up to 4 days without cleavage appearing. Such eggs will however cleave within a short time (15–30 minutes) after being restored to room temperatures. The cleavage is abnormal (similar to that recorded above) and gives evidence of permanent effects produced by the low temperature.

2. EFFECT OF TEMPERATURE ON LATER CLEAVAGES

In order to test the effect of temperature on cleavages after the first, the following experiment was undertaken. Eggs from the same female were fertilized and placed at the following temperatures: 5.3°, 9.0°, 11.9°, 14.9°, 17.9°, 21.3°, 25.0°, 26.8°, and 30.5°. At regular intervals samples were taken and fixed in 4 per cent formalin. These were subsequently examined after the membranes had been removed and the surface cells were counted under a binocular. At least ten eggs served as a sample in each case. Counts do not offer much difficulty up to the sixty-four cell stage. Beyond this stage, complications enter, owing both to the larger number of surface cells and to the occurrence of more frequent internal divisions. Counts were facilitated in such stages by fragmenting the eggs and counting each fragment separately. Such procedure agreed within 5 per cent with counts on unfragmented eggs.

Table 1 gives the average number of cells at the indicated time after fertilization for each temperature studied. The standard deviation of the cell number is also presented, to indicate the variability that may be expected. In studying these figures, greater relative variability will be noted among the earlier cleavages than among the later ones. The reason for this, however, may be attributed to the necessity of apportionment of the earlier cleavage stages among integral cell numbers, since an estimation of fraction of cell number, from the extent to which a furrow has been completed, is rather difficult. This variable diminishes when the cells become smaller and

more numerous. This same factor may also give a meaning to the odd cell numbers indicated among the average cell numbers in the table, namely, that these odd numbers may indicate the stage in the

TABLE I

EFFECT OF TEMPERATURE ON CLEAVAGE

Temp. (C.)	Time (Hr.)	No. of Cells	log n	σ	Temp. (C.)	Time (Hr.)	No. of Cells	log n	σ
26.8°	2.35	2.0	.30	.....	14.9°	4.98	1.5	.18	.....
	2.78	3.0	.48	.....		5.98	2.5	.40	.....
	3.37	7.7	.89	1.9		6.98	4.4	.64	2.0
	3.72	14.1	1.15	1.8		8.15	8.0	.90	.5
	4.22	24.1	1.38	1.7		10.23	27.8	1.44	3.9
	5.08	64.8	1.81	4.2		11.57	46.5	1.67	2.4
	5.63	97.5	1.99	12.3		13.90	71.8	1.86	4.3
25.0°	2.42	1.5	.18	.....	11.9°	7.63	1.5	.18	.....
	3.23	5.6	.75	1.5		8.45	2.0	.30	.....
	3.90	12.4	1.09	.9		10.47	3.6	.56	.7
	4.58	26.0	1.42	5.3		11.70	6.5	.81	1.2
	5.23	48.4	1.68	2.8		13.37	12.5	1.10	2.6
	6.07	91.8	1.96	12.3					
					9.8°	8.06	1.0	.00	.....
21.3°	2.45	0.0	.00	.....		20.00	21.5	1.33	3.5
	2.82	1.5	.18	.....		22.40	31.5	1.50	3.0
	3.48	2.0	.30	.....		27.00	85.6	1.93	11.2
	3.95	6.0	.78	.....					
	4.45	8.0	.90	.....	9.0°	9.20	1.0	.00	.....
	5.30	13.8	1.14	4.3		9.73	1.5	.18	.....
	6.03	33.2	1.52	10.4		12.45	1.9	.28	.....
	7.18	67.1	1.83	5.4		14.79	3.0	.48	.....
	7.68	109.1	2.04	6.4		25.82	22.0	1.34	4.2
						30.28	58.5	1.77	2.9
17.9°	3.45	1.6	.20	.....					
	4.03	1.9	.28	.....	5.3°	27.50	2.0	.30	.....
	4.63	3.8	.58	.....		40.80	5.1	.71	1.6
	6.20	12.2	1.09	1.9		56.30	11.0	1.04	2.2
	7.13	29.7	1.47	2.0		79.30	49.0	1.69	7.1
	7.75	53.4	1.73	3.9					
	9.22	102.4	2.01	10.9					

n—number of cells. σ—standard deviation.

completion of any particular cleavage. From this point of view, the figures represent rate of furrow formation more directly than rate of cleavage. Since the two processes are bound together, in most normal cases, we can speak of the latter in terms of the former. The weight of this factor in causing variability may, however, be readily recognized during the first cleavage, when, before the furrow has

quite reached the vegetal pole (completing the two-cell stage), the second cleavage furrow may begin to form in most cases; thus, a four-cell stage is present in the animal pole region, while the vegetal pole region is still undivided. This inequality in rate of nuclear and cytoplasmic division is more pronounced above 15° than below that temperature.

This factor must also be remembered in the following analysis of the rate of cleavage at the same and different temperatures. Before one can determine the effect of temperature on cleavage, it is necessary to determine how the rate of cleavage behaves with time, at constant temperature. The simplest assumption is that the number of cells being produced at any time is proportional to the number of cells present at that time. This is analogous to the mass law of chemical reactions. It signifies that, other conditions remaining constant, the rate of cell division is also constant. If x represents the number of cells at any time (t), this assumption may be symbolized by the equation:

$$\frac{dx}{dt} = kx \, .$$

The integral form of this relation may be expressed as follows:

$$\log x = kt + C \, ,$$

in which x and t are the same as before, while C represents the constant of integration.

A test of this assumption has been made by plotting the logarithm of cell number as ordinate and the time as abscissa (Fig. 2) from the data of Table I. Additional observations are recorded for a temperature of 9.8°, which is for a different batch of eggs. A straight line fits the points well up to the stage corresponding with the logarithm 1.8 (sixty-four cell stage or sixth cleavage). The early dividing egg tends to behave, therefore, like dividing unicellular organisms during the early growth-phases of any colony (yeast, Richards, 1928; *Bacterium coli*, Lane-Claypon, 1909; and *Paramecium*, Mitchell, 1929).

The problem becomes a bit more complex when one examines the separate data for division of cells in the animal and vegetal regions

of the egg. If special counts are made of the pigmented cells and the non-pigmented cells separately, one finds that they have different rates of division. The results of such a study are represented by the broken lines in Figure 2, for the temperature 17.9°. Similar results have been obtained for other temperatures. It is obvious, then, that the slope of the line obtained for total cell division is a resultant of the slopes of the separate lines for yolk and pigmented cell division.

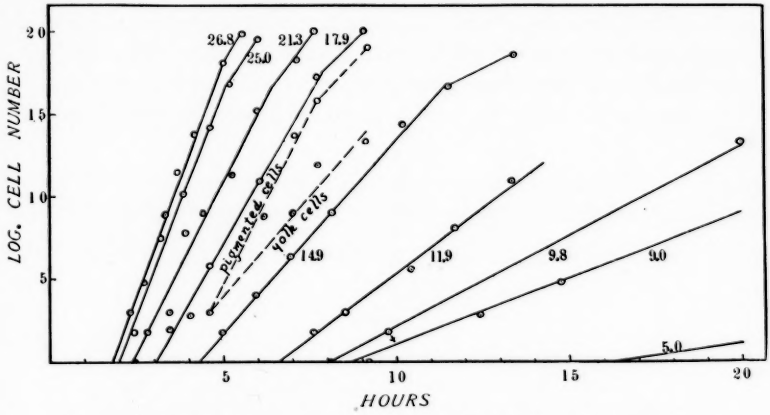

FIG. 2.—Isotherms of later cleavage. Data are from Table 1. The lines have been fitted by the method of least squares. The broken lines represent separate observations on pigmented and yolk cells at 17.9° C.

Despite this separateness in cleavage rates of the two components involved, the total rate of cell formation tends to remain constant.

There is a retardation, however, in the total cleavage rate at the sixty-four cell stage. This is due in part to internal cleavage, but also indicates a real decrease in rate of cell division. That a retardation must occur during cleavage is obvious from the fact that if the rate of cell production were to remain constant until the beginning of gastrulation there would be at least twenty-three cleavages (at 18°), with a resultant cell number of the order of one million. The actual order of magnitude, as estimated in several early gastrulae (from sections), is of the order of ten thousand cells. From this point of view it would be interesting to determine whether the rate of

retardation is continuous in later stages (as it appears to be in the studies of Richards on yeast cell-growth).

The time of appearance of injurious effects at each temperature extreme is a function of the intensity of heat treatment. Thus, at 29° the injury may be observed in many eggs only at a later yolk plug stage; at 30° they appear in the early gastrulae; and at 31° the cleavage stages are affected so that few gastrulate. At 32° the effect can be observed in the early cleavage stages, so that if eggs which have started to cleave are left for more than 6 hours at this temperature they die as early blastulae. Such eggs swell enormously, and the pigmented cells take on an appearance observed in centrifuged eggs: the pigment occupies the center of the cell with a whitish mass

TABLE II

INCREASE IN HEAT TOLERANCE DURING CLEAVAGE

Time (Hr.)	Exposure (Min.)	Time (Hr.)	Exposure (Min.)
1	45	5	190
3	60	6	300
4	150	7	400

Time = hours after fertilization when exposed to 32.5° C. Exposure = minimum to produce 100 per cent abnormal embryos. Controls at room temperature showed 5 per cent abnormals.

around the periphery. At both temperature extremes, but particularly observable at lower temperatures, cleavage furrows may be formed but may disappear later. The yolk cells are more susceptible to these injurious effects than the pigmented cells.

Susceptibility to heat treatment is counteracted to a certain extent by a progressive increase of tolerance to elevated temperatures. To test this the following experiment was performed. Fertilized eggs were left for varying lengths of time at 32.5°. They were removed and permitted to develop at room temperature (21°). The resulting embryos included varying numbers of abnormal animals. In Table II the results are summarized, and they are plotted in Figure 3. The decrease in susceptibility follows the increase in cell number and is approximately proportional to the number of cleavages. This would be in harmony with a gradual conversion of yolk to cytoplasm as the potent factor in the increased tolerance.

3. EFFECT OF TEMPERATURE ON GASTRULATION

In order to study the conditions which lead to the process of gastrulation, a study has been made to ascertain the effect of temperature on the time necessary for the dorsal lip to become visible. The end-point of this reaction has been taken as the time when the beginning of invagination is foreshadowed by the concentration of the pigment in the boundary between yolk and pigment cells. This can be determined within 15 minutes at temperatures above 15°, within ½ hour between 9° and 15°, and within 10 hours below 9°. In Figure 4 are plotted the data obtained in this study. Abscissae represent tem-

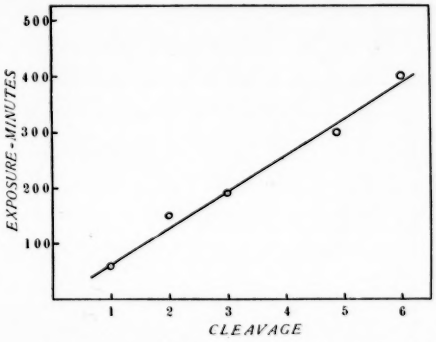

FIG. 3.—Increase in tolerance to a temperature of 32.5° C. during cleavage. The ordinate at each point represents the minimum exposure necessary to produce abnormalities in 100 per cent of the embryos.

peratures; ordinates represent the times necessary for 50 per cent to complete the reaction. The temperatures vary from 9° to 29°. Beyond these limits more than 50 per cent of the eggs fail to gastrulate; and those that do, vary greatly in the time necessary. The time for these extreme temperatures represents that within which the first ones gastrulate. The limits of this process are in the neighborhood of 5° and 33°. In one experiment—on eggs at 4.5°—only pigment concentration appeared without invagination, after 1 week; the controls, at 20°, gastrulated in 2 hours. At both temperature extremes eggs may gastrulate, after many of the yolk cells have been injured and appear vacuolated. Above 30° the gastrulation may occur while the pigmented cells are still quite large. Such gastrulae however do not form ventral lips, and die soon.

4. EFFECT OF TEMPERATURE ON LATER PROCESSES

Processes beyond this point become more difficult to analyze, since growth, as well as cleavage, begins to take place. This introduces a greater amount of variability than is met with during preceding stages. Some batches of eggs are more homogeneous, in this

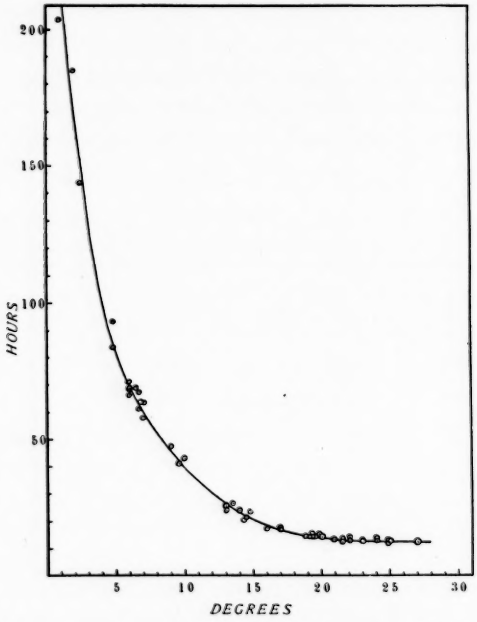

FIG. 4.—Temperature-time relation for beginning of gastrulation. Each point represents an average of data from eggs of one female.

regard, than others; and as far as possible, subsequent results are restricted to such batches which tended to be more homogeneous. Another difficulty is the choice of suitable end-points, which can be determined within a short time after their occurrence. The problem has been attacked by picking a morphological stage at which a specific process has been completed, and by comparing the times necessary to reach the stage at different temperatures. Another complexity enters here. Although different parts of the embryo re-

Page 300: Fig. 4. On the abscissa axis, substitute for the numbers: 5, 10, 15, 20, 25, the numbers below:
9, 14, 19, 24, 29.

spond in their reactions to temperature synchronously, some derangements of rates of processes have been observed. These will be alluded to in their proper place.

Stages which have been chosen for study are the following:

1. The first signs of invagination at the ventral lip (represented by fusion of the lateral lips).
2. The formation of the slit blastopore.
3. Fusion of the neural folds (beginning).
4. Growth stages—stages in definite tail length.
5. Completion of the first metamorphosis, as represented by the completion of the backward growth of the operculum over the external gills.

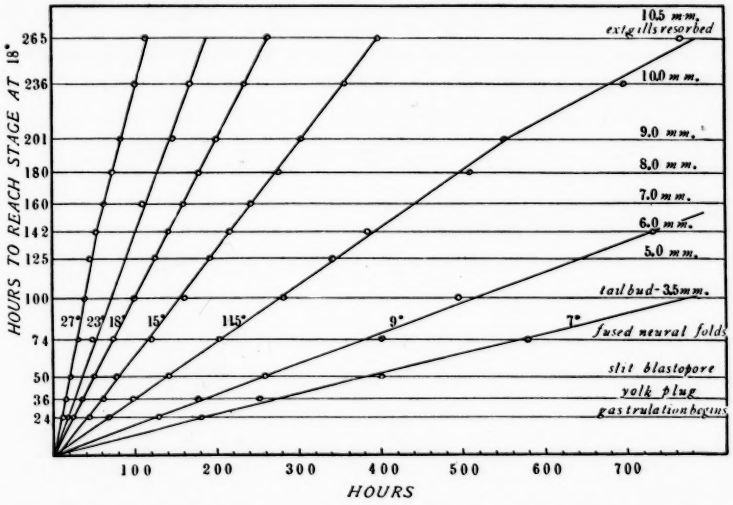

FIG. 5.—Isotherms of development. The ordinates represent time relations of different stages at 18° C. Each point is an average of observations taken from different females.

To co-ordinate these results in a graphical manner, as well as for purposes of comparison with other workers' results, they have been plotted in Figure 5 in the following manner: Time in hours after fertilization is the abscissa. Time (hours after fertilization) for eggs at

18° to reach any stage is the ordinate. As a result of the choice of co-ordinates, the plot of the course of development at 18° yields a straight line. The observations at other temperatures were plotted with this as a basis. This manner of plotting enables one to determine the interrelations between rates of development of different stages at the same temperature. It also helps one correlate such interrelations between different stages with rates of development of the same stages at different temperatures. It will be seen from Figure 5 that the points at temperatures other than 18° can be fitted closely with a straight line, the slope of which remains constant over a considerable period of development. This relation indicates that change of temperature modifies the rates of development of the embryo, during different stages, in a constant manner. In mathematical terms, if rate of development, as expressed by $(\delta v/\delta x)$, is a function of age (x) and temperature (t), other conditions remaining constant, then at any temperature (t)

$$\left(\frac{\delta v}{\delta x}\right)_t = f(x) .$$

At any other temperature $(t+a)$

$$\left(\frac{\delta v}{\delta x}\right)_{t+a} = kf(x) .$$

The deviations from this generalization which occur are, for the most part, those that take place beyond the period of primary differentiation or determination. In those cases in which deviations occur, they are accompanied merely by a change in the constant k. In practically all points the deviation of the observed values from the fitted lines is not greater than 10 per cent which is within the limits of variability observed in different batches and in some cases in the same batch of eggs.

5. EFFECT OF TEMPERATURE ON SIMULTANEOUS PROCESSES IN DIFFERENT PARTS OF THE EMBRYO

Mention has already been made of the different rates of division of yolk and pigmented cells. The yolk cells are also more susceptible to heat injury. This is responsible for most of the abnormalities en-

countered in the early embryo during and after gastrulation. One such variation, encountered at higher temperatures, is the occurrence of invagination at several places simultaneously, along the boundary between yolk and pigmented cells. Another is the occurrence of half-embryos and ring embryos, of various types, which have been adequately described and pictured by workers on various species of *Rana* (Hertwig, 1898; Morgan, 1905; etc.). The differences in susceptibility can be brought out markedly by the following type of experiment. Eggs can be kept as early gastrulae for several days at 5°, and then can be placed at 25° or above. The pigmented cells will then continue rapid development and the neural folds will soon appear, while a large yolk plug is still apparent externally. The area which appears to be most sensitive to extremes of temperature is that part of the egg in which the ventral lip is to appear. In the tolerance experiments mentioned above (p. 298), at 32°, the main abnormality produced after short exposures was the characteristic cytolysis in this region. These injurious effects are entirely similar to those produced by centrifuging, salts, and lack of oxygen effects, reported by various workers. They may all be summarized as interfering with the overgrowth of the yolk plug by the pigmented cells. The effect of temperature on the rate of overgrowth of the pigmented cells is indirect in this case, since the rate of growth in other directions seems to be unhampered. Injury to the yolk cells may erect some barrier or remove some substance which accelerates the growth of the cells around the yolk plug. That an injurious effect on the pigmented cells is not involved may be noted from the fact that the overgrowth of the yolk plug may continue, at a slower rate, until the entire plug is covered. Such embryos may survive a long time although because of the retarded growth, duplications of the tail may arise.

Effects due to actual differences of growth rate, and not to injury, have been noted after a quantitative study of the rates of development of different parts at different temperatures. In several batches of eggs measurements have been made of body length, tail length, and gill length in the same embryos. These have served as the basis for the points in Figure 5. The observations on the eggs from a single female have been plotted in Figure 6. The eggs were placed at the temperatures indicated shortly after natural fertilization. All

measurements have been made by means of an ocular micrometer (6× ocular, 33-mm. objective). Ordinates represent lengths in millimeters; abscissae represent times after fertilization. Body length = from tip of head to anus; tail length = from anus to tip of tail; gill length = from point of attachment of the first external gill, along its

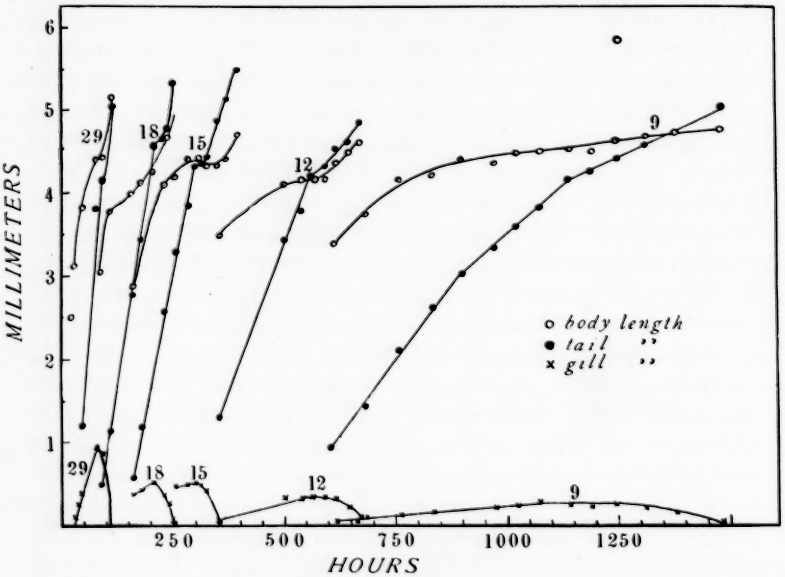

FIG. 6.—Growth of body, tail, and gill at different temperatures. Observations represent averages from five to ten embryos. All eggs were derived from the same female. The numbers above the curves refer to temperature.

axis, to the end of the longest filament. Each observation is the average of five to ten individuals.

It will be seen that the general shape of the curves for each temperature is similar. Increases and changes of growth rate are paralleled throughout. Body length increases up to 85 units and remains constant, or may undergo a slight decrease for a short while. Tail length undergoes an increase which is apparently constant for a considerable interval of time, but then falls off. Gill length increases

to a maximum, and then declines. The decline in size of the external gills, at this time, is associated with the backward growth of the operculum, which gradually covers the gills, so that they appear shorter.

The greatest difference, in relative growth, owing to temperature effects, is in the rate at which the gills grow. There is a gradual increase in the maximum size of gills as the temperature is increased.

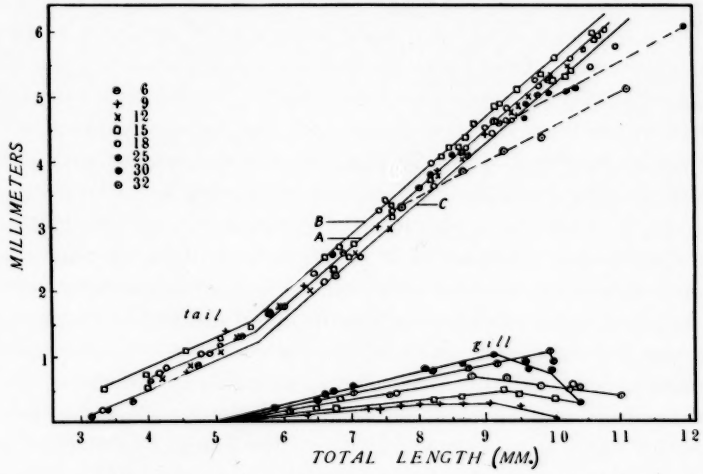

FIG. 7.—Growth of tail and gill relative to total length of body at different temperatures. The lines represent observations at separate temperatures: *A*, 18°; *B*, 15°; *C*, 30°.

That this is due to a more rapid increase rather than to a longer period of growth is indicated in Figure 7. Here is plotted the size of gill as ordinate, and the total body length (tip of head to tip of tail) as abscissa. In this figure it will be seen that at the higher temperatures the size of gill for any particular body size is greater than that at the lower temperatures. Furthermore, the decrease in gill length commences at a body size which is the same, approximately, for all (about 9 mm.). In the same figure the length of tail against body length is also plotted. In this case the combined data for observa-

tions from four different females at different temperatures has been plotted. The relative increase of tail length in eggs at the same temperature are indicated by the symbols which are defined in the legend. The points fall within two series of parallel lines. In the younger embryos the slope of these lines is less than in the older embryos. The change commences at a total body length of 5.5 mm. Before this point, tail and body are contributing to increase in total length. After this point increase in body length is greatly diminished. (There is a greater increase along other axes of the body at this time, however.) The increase in total body length after this point is due principally to the increase in tail length. This is indicated by the slope of the lines, which is almost 45°. The series of parallel lines indicated have been drawn through points corresponding with eggs from the same female at the same temperature. Line *A* is for a series at 18°, *B* is for one at 15°, and *C* for one at 30°, all for different batches of eggs. Comparison of results for eggs from the same and different females, at different temperatures, shows a close agreement, over a wide range, in relative growth rate of tail and body length. In two series observed, however, the embryos at 18° C. showed a greater relative tail size than embryos at higher or lower temperatures. However, the data are not complete enough to indicate whether the differences are significant. The point to be stressed is that the relative growth rate is constant, except for the highest temperatures studied (30° and 32°)—and for these the deviation occurs only later in the growth phases studied. Curiously, the growth rates in these changes to a value approximately similar to that observed during the first stages of growth.

DISCUSSION

For the analysis of the effect of temperature on development, in the past, various equations have been employed by different workers (Peter, 1905; Krogh, 1914; Crozier, 1924; Janisch, 1925; and Belehradek, 1930). Each of these has its advantages and defects. For representing the data presented in this paper, the following relation has been assumed.

Let V = velocity and T = absolute temperature. Then

$$\log V = f\left(\frac{1}{T}\right).$$

In its simplest form this equation reduces to the Arrhenius equation, so widely used in theoretical analyses of velocities of chemical reactions. Other forms may be compared with this by plotting the reciprocal of temperature against the logarithm of velocity. This has been done for the different stages of development in Figure 8. (Velocity in each case denotes either the reciprocal of time or the slope of a particular isotherm taken from Figures 2 and 5.) To include the extreme points, at any temperature, parallel curves have been fitted indicating the variability of each stage.

It will be seen that at the lower temperatures (5°–15°) the points of all the stages fall within two parallel curves. Over the intermediate temperature range (10°–20°) the points indicate a flattening of the curves, with a possibility that a straight line may fit the data over this range. The average change of velocity with temperature in this region indicates the possibility of a constant energy of activation equal to 24,000 calories per mol. At the higher temperatures the separate data diverge and indicate different limits of variability for the different stages. A definite order seems to be maintained, in that the cleavage data show smaller increases of velocity with rise of temperature than do the gastrulation data, while the latter show a similar relation (at a slightly higher temperature) to the data of later development.

This method of representation is chosen because it links together the different stages and offers evidence for a common basis of reaction to temperature for the successive phases of development. Whether any limiting reactions, according to the interpretation of Crozier (1924) are involved, cannot be ascertained from the data, which does seem to indicate, however, that if there are such reactions they may be common to the different stages. Indications of similar conditions have been put forth by Worley (1933) for the mackerel. The divergence which occurs at higher temperatures may possibly be correlated with the increased heat tolerance which occurs as development progresses.

SUMMARY

1. A study has been made of the effect of temperature on the rates of different processes of early development of *Rana pipiens*, including first cleavage, later cleavage, gastrulation, neural fold formation, and early growth.

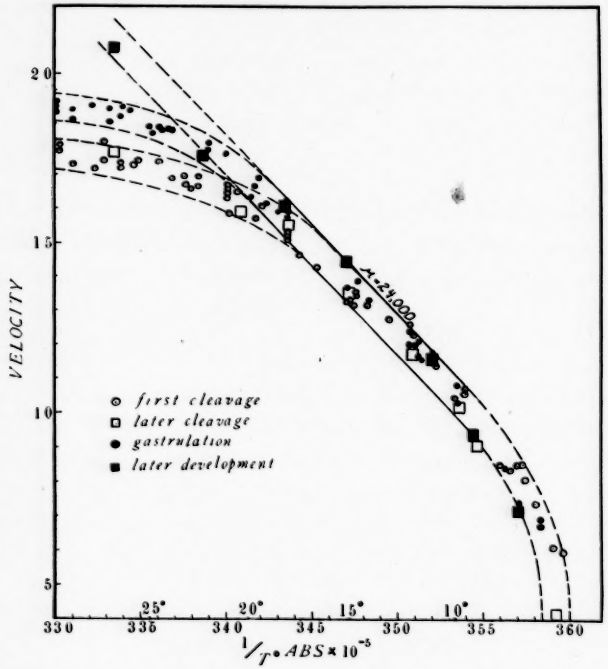

FIG. 8.—Relation between the reciprocal of absolute temperature and the logarithm of velocity.

Stage	*Velocity*	*Data*
First cleavage	reciprocal of time	Fig. 1
Later cleavage	slope of isotherm	Fig. 3
Gastrulation	reciprocal of time	Fig. 4
Later development	slope of isotherm	Fig. 5

μ = the constant of the Arrhenius equation equivalent to the energy of activation.

2. The change of susceptibility to heat injury has been studied for the cleavage stages. An increase in tolerance to heat, proportional to the number of cleavages, has been found to occur.

3. The cleavage rate remains constant up to the sixty-four cell stage.

4. The rate of development during any stage after gastrulation is a function of that stage and a constant determined by the temperature.

5. A study has been made of the relative rate of growth of body, tail and gill at different temperatures. The relation of gill growth to body growth is changed by temperature in such a way that the maximum size attained by the external gills decreases as the temperature decreases.

6. The increase in relative velocity of development with increase in temperature over the lower temperature range is identical for the different stages of development. At the higher temperature range there is a less rapid increase in relative velocity for the earlier stages as compared with the later stages.

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THE RELATION OF TEMPERATURE TO RESPIRATORY REGULATION IN THE LEECH (HIRUDO MEDICINALIS)

(One figure)

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THE influence of various environmental factors on the respiratory metabolism of aquatic invertebrates has attracted the attention of numerous investigators. Those of especial interest concern the relationship of oxygen consumption and oxygen tension. Evidence has been presented through the works of Lund (1921) on *Planaria;* Hyman (1929) on echinoderms; Hyman (1932), *Nereis;* Amberson, Mayerson, and Scott (1924), lobster and *Nereis;* Helff (1928), crayfish; Galtsoff and Whipple (1930), oyster; Nazawa (1929), oyster; Hiestand (1931), crayfish; and Lindeman (1932), leech, which indicates that there exists no general uniformity among aquatic invertebrates relative to their ability to regulate oxygen intake when subject to varying oxygen tensions. When attempts have been made to compare the respiratory ability of various invertebrates, the temperatures employed in the different investigations have not been correlated. Among the papers listed above, for example, the following temperatures were employed: Lund, 20° C.; Amberson *et al.*, 21° C.; Helff, 25° C.; Hyman, 20° C.; Galtsoff and Whipple, 24.5° C.; Heistand, 19.5°–22° C.; Lindeman, 25° C. If any attempt is to be made in comparing aquatic invertebrates relative to respiratory regulation, it seems desirable that a uniform temperature be employed. However, before any generalizations can be made, it should be determined to what extent alterations in temperature affect respiratory regulation.

The present investigation was undertaken for the purpose of ascertaining to what extent variations in temperature influenced the ability of the leech to regulate oxygen intake at various oxygen tensions.

MATERIAL AND METHODS

The leech, *Hirudo medicinalis*, obtained from the General Biological Supply Company in Chicago, was used in all experiments. They were medium-sized, healthy specimens measuring about 3 inches in extended position. No data were available upon when they had last taken food; so upon arrival they were placed in large aquarium jars for at least 3 weeks before being used for experimental purposes. This procedure allowed sufficient time for any food in the digestive tract to be digested, and thus eliminate any error from this source.

The apparatus employed for the determination of oxygen consumption consisted of a 4-liter flask. Into the neck was fitted a rubber stopper through which extended two inlet tubes and a glass stirring device. Into one inlet tube a separatory funnel was provided for replacing water which had been withdrawn for oxygen analysis. A glass outlet tube for withdrawing water extended into the flask through a neck provided in the side.

In brief, the experimental procedure was as follows: The flask was filled with tap water and placed in a bath kept constant to 0.1° C. until the temperature of the water was approximately that of the bath. From twenty to twenty-five animals were then placed in the respiratory chamber, and the stopper sealed with vaseline. The animals were allowed to remain in the flask several hours before a sample for testing was withdrawn. This provided sufficient time for the animals to become acclimated and insured normal respiration. Approximately 60 cc. of water was withdrawn and analyzed for dissolved oxygen. The water withdrawn was immediately replaced with fresh water of approximately the same oxygen content. At various intervals throughout the course of the experiment the same procedure of testing the oxygen content of the water was followed until the animals had reduced the tension to the desired level.

The Winkler method was employed for determining the concentration of dissolved oxygen in the samples of water, and 0.04 N sodium thiosulfate was used for all titrations. The temperatures used during the course of the experiment were as follows: 10° C., 17° C., 24° C., 30° C., 34° C.

RESULTS AND DISCUSSION

That changes in temperature exert a marked influence upon the rate of oxygen consumption of animals is well established. However, to the author's knowledge no direct data are available regarding the relation of temperature to the amount of oxygen consumed at different oxygen tensions. The data reported in this paper are based upon approximately fifty experiments employing five different temperatures. The results of a single series of experiments employing the same group of animals throughout are recorded in Table I.

TABLE I

CONSECUTIVE DETERMINATIONS OF OXYGEN TENSION IN TERMS OF CUBIC CENTIMETERS PER LITER OF WATER AT FIVE DIFFERENT TEMPERATURES. TWENTY-TWO ANIMALS

Hours	Temperatures				
	10° C.	17° C.	24° C.	30° C.	34° C.
1	3.75	3.63	4.10	5.04	4.86
2	.....	.....	3.60	4.31	3.51
3	.....	3.12	3.15	3.55	2.11
4	3.04	.....	2.60	2.88	*1.38*†
5	.....	2.50	.....	2.18	0.94*
6	2.65	.....	1.57	*1.35*†	0.73*
7	.....	1.99	*1.09*†	1.11	.....
8	2.25	.....	0.78	0.80	.....
9	.....	1.52	0.60	.....	.....
10	.....	.....	.....	.....	.....
11	.....	1.03	.....	.....	.....
12	1.56	*0.78*†	.....	.....	.....
13	.....	0.58	.....	.....	.....
14	1.23	.....	.....	.....	.....
15	.....	.....	.....	.....	.....
16	0.87	.....	.....	.....	.....
17	0.69	.....	.....	.....	.....
18	*0.42*†	.....	.....	.....	.....
19	0.39	.....	.....	.....	.....
20	0.34	.....	.....	.....	.....

* Indicates half-hour intervals. † Indicates critical tensions.

From the table it is to be noted that the oxygen consumption for each temperature is relatively constant from hour to hour within a certain range of oxygen concentration. However, it will be further noted that the range of respiratory regulation became greater as the

temperature was decreased. The data obtained in these experiments can best be represented in graphic form as shown in Figure 1.

The graph represents a series of five experiments using the same temperatures as in Table I but a different group of animals. The

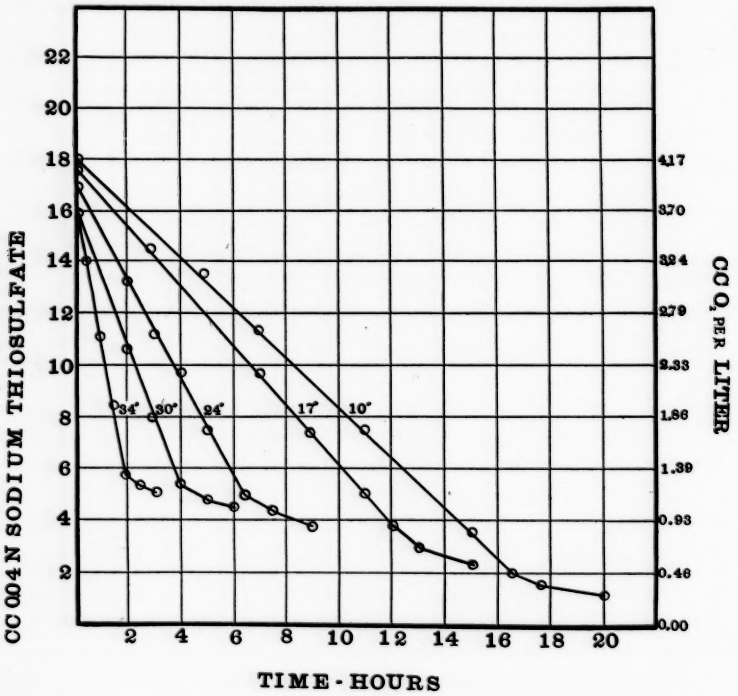

FIG. 1.—Curves showing the ranges to which oxygen consumption is independent of oxygen tension for temperatures of 10° C., 17° C., 24° C., 30° C., and 34° C.

points on the graph represent the number of cubic centimeters of 0.04 N sodium thiosulfate required to titrate the dissolved oxygen in the samples of water taken from time to time as the animals reduced the oxygen tension by their own respiration. An inspection of the graph reveals essentially the same situation as shown in Table I, namely, the existence of a linear relationship between oxygen con-

sumption and oxygen concentration above a critical tension which shifts with change in temperature. The critical tensions found in this particular series of experiments were approximately as follows: 1.31, 1.25, 1.12, 0.84, 0.46 cc. of oxygen per liter at the following temperatures 34°, 30°, 24°, 17°, 10° C. The results obtained in this series, as well as in all the others, clearly indicate a greater range of respiratory regulation in the leech at low temperatures than at a high.

The term "critical tension" referred to above designates the concentration of dissolved oxygen below which oxygen consumption becomes a function of oxygen concentration.

TABLE II

AVERAGE CRITICAL TENSIONS

Temperature	Oxygen per Liter (Cc.)
10° C.	0.45
17° C.	0.85
24° C.	1.19
30° C.	1.31
34° C.	1.34

The complete data for all experiments have been compiled with reference to the average critical tensions for each temperature, and these means are presented in Table II.

Attention should be directed to the relatively large difference in critical tension between 10° C. and 24° C., as compared to that between 24° C. and 34° C. An inspection of the table reveals a difference of 0.72 cc. oxygen per liter between 10° C. and 24° C., as compared to 0.15 cc. oxygen per liter between 24° C and 34° C.

The results as described above for the leech *Hirude medicinalis* indicate the importance of temperature in experiments involving the relationship between oxygen consumption and oxygen tension. As a general rule, experiments involving measurements of respiratory exchange are conducted at temperatures approximating that of the laboratory. These temperatures are usually higher than the normal

environment to which the animals are accustomed. Based upon the results presented in this paper, it may be assumed that the measurement of respiratory requirement of animals under conditions other than those encountered normally in the environment may not always be a true test of their ability to regulate oxygen consumption. The question which naturally suggests itself is whether or not certain invertebrates which have shown a relatively narrow range of respiratory regulation under experimental conditions would not show a greater range if tested under temperatures similating more nearly those of the environment.

SUMMARY AND CONCLUSIONS

1. The rate of oxygen consumption in relation to oxygen tension has been determined for the leech, *Hirudo medicinalis*, at the following temperatures: 10°, 17°, 24°, 30°, 34° C.

2. The concentration of dissolved oxygen below which oxygen intake becomes a function of concentration has been designated as "critical tension."

3. The range to which oxygen consumption is independent of oxygen concentration has been found to be considerably influenced by temperature, i.e., low temperatures being more conducive to a greater range of respiratory regulation than high temperatures.

4. From the data obtained on the leech, evidence is presented which indicates that experiments conducted at relatively high temperatures do not always give conclusive evidence of the animals' ability to regulate oxygen consumption at low oxygen tensions.

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VARIATIONS IN THE RESPIRATORY METABOLISM OF THE PACIFIC KILLIFISH FUNDULUS PARVIPINNIS DUE TO SIZE, SEASON, AND CONTINUED CONSTANT TEMPERATURE

(Three figures)

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IT IS known that various species of fishes live in waters of differing temperatures, ranging from 0° in the northern latitudes to 30°[1] and above in the tropics. It is also known that fishes from warm waters of the tropics, when placed in water below 15°, soon perish, and that fishes from cold water are quickly killed when placed in warm water. In fact, the phenomenon of partial acclimatization to temperature may be demonstrated in the laboratory with *Fundulus parvipinnis*. If these fishes are transferred directly from water at temperatures below 15° to water at 34°–38° they are killed in from 2 to 5 minutes, and if fishes which have been gradually adjusted to the higher temperatures and kept there for a period of 10 days or over are transferred directly to water at a temperature below 15° they are unable to adjust themselves to the cold water and invariably die in from 13 to 36 hours, depending on the temperature.

One would expect that fishes which live at a very low temperature would show a relatively higher rate of metabolism at this low temperature than would other fishes living normally at a higher temperature. For example, fishes living normally at 10° should have a higher rate of metabolism at this temperature than would fishes which live normally at temperature above 20° if they were suddenly lowered to 10°, provided that the latter could survive the change. Montuori (1907) attempted to acclimatize fishes to warm water by gradually heating the water in the aquarium in which they were kept, thus raising the temperature from 11°–13° to 27°–30° over a period of several days. After 2 days, he compared their oxygen con-

[1] All temperatures are given in degrees Centigrade.

sumption at the high temperature with that found at the low and found that it had not increased, but actually decreased! Obviously the fishes were either no longer normal or there was a very serious error in his determination of their metabolism.

The only reference I find in the literature relative to the influence of season on the rate of metabolism of fishes is Cronheim's (1911) analysis of Knauthe's experiments, from which Cronheim concludes that the metabolism of the carp is not only dependent upon the temperature but on the time of year. He finds that the oxygen uptake is less in the fall than in the spring and attributes this to the probability that in the autumn the fishes are beginning their period of winter rest. He further states that "da in dieser Zeit keine oder nur geringe Nahrungsaufnahme stattfindet so wird naturgemäss ein Herabgehen des Stoffwechsels stattfinden." That Knauthe's data indicate a possible seasonal variation in metabolism is certainly shown, but Cronheim's conclusions that the fishes are entering a period of winter rest do not seem to be warranted. I have studied carefully Knauthe's data relative to the point in question. The values given were obtained during the months of April, May, June, July, August, September, and October. Those for July and August were at a much higher temperature and could not be used for comparison. While the average values for the months of April, May, and June are higher than those for September or October, there is no indication of a winter rest. The average values for October are 28 per cent higher than for September, while the values for the first three months are only 21 per cent higher than for October. The increased metabolism in October over that of September is significant, and the mere fact that the metabolism during these two months is lower than in the spring does not preclude the possibility that in these fishes the metabolism in winter at a given temperature is higher than it would be in summer at the same temperature.

Since it seemed reasonable to suppose that fishes living normally at 10° or below have a higher rate of metabolism at this temperature than do fishes which live normally above 20° and are suddenly lowered to 10°, the purpose of this investigation was to endeavor to determine, by acclimatizing *Fundulus* to waters of high and low temperature, whether or not their rates of metabolism could be changed

to a reasonable extent, and whether or not there are seasonal variations.

In pursuing a program of this nature it is, of course, necessary to know what the relation is between temperature and the rate of metabolism of fishes of a given size or age. Furthermore, it is necessary to determine the relation between rate of metabolism and the size or weight of fishes of a given species at a constant temperature, and whether this relation is constant over the normal temperature range. That is to say, will the amount of oxygen consumed by two sets of fishes of the same species, but differing considerably in size vary by the same amount at a low temperature as at a high one? I have discussed certain of the underlying relations (Wells, 1932, 1935) in other publications, and presented data to show the relation of temperature to metabolism in *Fundulus parvipinnis*.

MATERIALS AND METHODS

In a previous publication in this journal I have described the apparatus used for the determination of oxygen consumption of fishes (Wells, 1935). Briefly, it provides a continuous-flow, constant-temperature system, in which the rate of flow can be maintained at any desired value, and the temperature can be controlled to within 0.1°. After the fishes are placed in the respiratory chamber they are in no way disturbed until an experiment is completed. In all cases where the results from two or more sets of data were to be compared, the interval after the time of feeding was the same, a fact which eliminates any variation due to differences in the nutritional state.

The oxygen was determined by the Winkler method, which is so generally in use that it does not require discussion. It should be emphasized, however, that the amount of nitrate present in the sea water, even when the fishes are forced to use one-half of the available oxygen, is so small that no modification of the Winkler method is necessary. The addition to the sea water of 0.03 mg. of nitrite per L. expressed as nitrogen had no effect on the end-point in the Winkler titration. It was found that under the most unfavorable experimental condition the greatest amount of nitrite present was 0.004 mg./L. (expressed as nitrogen).

RELATION OF SIZE TO METABOLISM

It is extremely difficult to raise marine fishes in the laboratory, hence the relation of age to rate of metabolism must be determined on the basis of size. This is justified because I have found that the rates of growth of individual fishes kept in the laboratory agree fairly closely with one another if all have been kept at the same temperature. That is to say, if one selects a number of very small fishes weighing approximately 1 gram each, keeps them under the same conditions, and is so fortunate as not to lose them through disease, at the end of any given period each fish will have increased almost equally in weight. A group of twenty-nine small fishes weighing on an average of 1.8 grams each were selected from a catch brought into the laboratory in February, 1932. Seven of them which survived the disease epidemic during 1933 (Wells and ZoBell, 1934) weighed on an average 13.4 grams in February, 1934. Of these seven the smallest, a male, weighed 12.8 grams, the largest, a female, weighed 14 grams. Those that succumbed to the disease were of the same size as the survivors at the time of their death. It may be stated that the seven fishes which survived were larger, without exception, than any obtained in the catches brought in from the bay and probably represent the maximum size attained by this species in this locality.

Experience has shown (DuBois, 1927) that the most satisfactory method of comparing basal metabolism of warm-blooded animals is heat production per square meter of body surface, but there is no valid reason for assuming that the same rule should hold for fishes, since in general their body temperatures are virtually identical with that of their environment. That the surface area law for homoiotherms does not hold for cold-blooded animals seems to be accepted (Dhar, 1926; Terroine and Delpech, 1931). Rubner (1924) concludes that it is not valid for poikilotherms: "Die Oberfläche bleibt als regulierendes Prinzip nur für den Energieverbrauch des Warmblüters. Bie teifer stehenden Tieren hat sich eine solche Beziehung nicht finden lassen, im Gegenteil, man kann sagen, die Oberfläche hat noch keine kausale Bedeutung für den Kraftwechsel."

Very few previous experiments have been attempted for the purpose of studying the relation between size and oxygen consumption, and I am aware of none that were carried out at a constant tempera-

ture, in which fishes of one species were utilized over practically their entire weight range. The conclusions which have been drawn from the few available experiments are conflicting and uncertain. The experiments of Knauthe (see Cronheim, 1911), Montuori (1913), and Lindstedt (1914) may be cited as illustrations. Cronheim concludes that the oxygen metabolism of carp is proportional to the body surface, but his values are so variable that it is hard to see how he arrived at his conclusions. Montuori concludes from his results on fishes and other marine animals that metabolism is proportional to body weight. Lindstedt finds considerable variation in the relation of metabolism to body surface in tench, and an enormous difference when he compares fishes of different species. The oxygen consumption of rainbow trout in relation to body surface was five times as great as for tench. Concerning the relation of metabolism to body weight, body surface, and size in reptiles, we have much more definite information. Benedict (1932), in his excellent monograph on the physiology of large reptiles, has calculated metabolism both on the basis of body weight and surface area. He finds that there is little choice between surface area and body weight as a means of comparing reptiles of different species. So far as relation of metabolism to size in any given species is concerned, his results are unsatisfactory. Considering all the species with which he worked, the smallest animals have the highest metabolism per kilogram of body weight. On the basis of surface area, however, the results are conflicting.

Keys (1931), using *Fundulus parvipinnis*, has constructed a curve showing relation of weight to metabolism from determinations on single fishes. These data were obtained from thirty-eight animals ranging in weight from 1.2 to 11.2 grams and over a temperature range from 14.8° to 22.2°, and have been "corrected" to a temperature of 20°. These results are open to criticism from two standpoints. First, as I have shown (Wells, 1932), the metabolism of this species of fish usually does not attain a normal rate until 24 hours after transfer of the animals to the respiratory chambers, and any values obtained in the first 8–10 hours may be much too high. To make this more clear, I shall give a concrete example. From my graph, Figure 2 (Wells, 1932), it may be seen that the oxygen uptake at 16° of twenty-nine fishes averaging 1.86 grams each, was 0.250 cc. of

oxygen per hour per gram in the interval from 6 to 12 hours after transfer to the respiratory chambers, while after 24 hours the average consumption had fallen to 0.163. Keys (1931) finds a fish weighing 1.9 grams to have an oxygen uptake of 0.254 cc. per hour per gram at 16.1°, but it is quite clear that the value 0.254 does not represent normal metabolism for fishes of this weight. It is much too high and a curve constructed from values obtained at various temperatures in this critical period (4–12 hours) to represent the relation of size to metabolism would not indicate the true relations.

The second criticism to be made of Keys's results, as I have shown elsewhere (Wells, 1935), is that the oxygen consumption of fishes of different sizes does not increase equally with increasing temperature. Therefore, to use the same temperature correction for fishes of different weights will distort the relation between metabolism and size.

I have completed a series of experiments on *Fundulus* to determine the relation of size to oxygen uptake at a constant temperature of 16°. With a single exception, every point on the curve (Fig. 1) represents the average oxygen uptake of twelve or more fishes[2] for a period of at least 10 hours, commencing 24 hours after transfer of the animals to the respiratory chambers. The fishes in each weight group were as nearly equal in size as it was possible to select. In many cases this was accomplished by weighing each fish separately. The determinations for all groups were made after the same interval from feeding, to insure a comparable state of nutrition, and, finally, the diet in all cases was the same.

The oxygen consumption of the small fishes is quite variable, particularly of those between 1.5 and 2.5 grams. This is to be expected for two reasons. It is very difficult to select a group of eighteen or twenty fishes of exactly equal size, even when one has a large number from which to choose. Also it is very difficult to weight live fishes to an accuracy of $\frac{1}{10}$ of a gram and, hence, when one is dealing with fishes from 0.6 to 2.5 grams, it is possible to select two groups of equal weight, but with more large fishes in one group than in the other. When this is the case, it is quite obvious that the metabolism of the two groups might differ considerably. This variation in size,

[2] The value for the 13.4 gram point was obtained from seven individuals, as these were all that were available in this weight group.

which cannot be avoided in the very small animals, is probably the cause of some of the variation in the range from 0.6 to 2.0 grams.

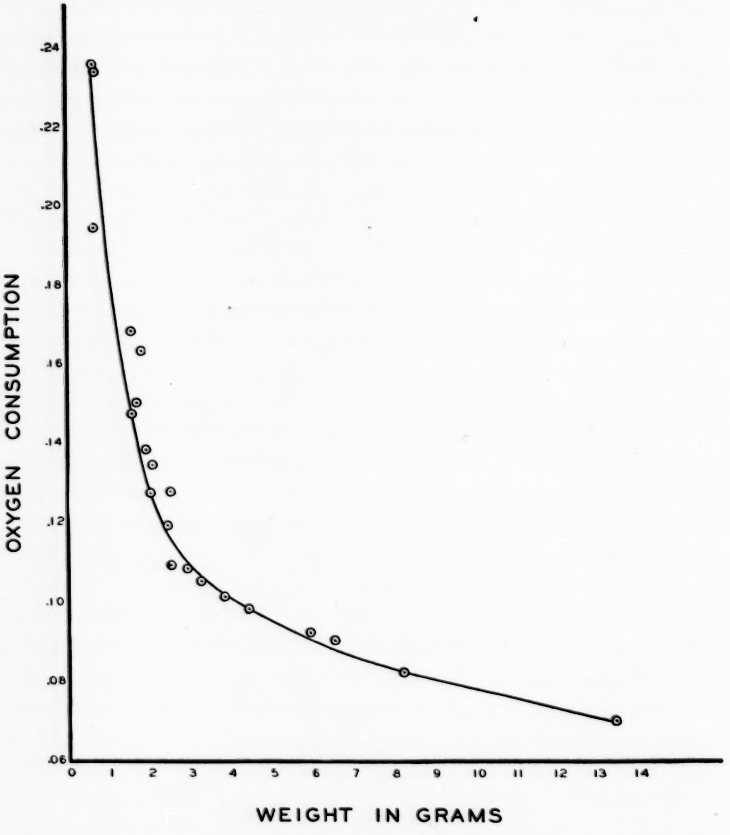

FIG. 1.—Relation of size to metabolism at 16° C.

Each point on the curve represents the average uptake of twelve or more fishes (values for 13.4 gram fishes were obtained from seven individuals) for a period of at least 10 hours commencing 24 hours after transfer of the animals to the respiratory chambers. The fishes in each weight group were as nearly equal in size as it was possible to select.

The second source of variation is the time element. Most of the values for the small fishes were secured during the months of Janu-

ary, February, and March, a period during which, as I shall show, a great deal of change takes place in the oxygen utilization of these animals.

The only significance of this curve is that it indicates approximately the relation between size and rate of metabolism at 16° in *Fundulus parvipinnis*, 24 hours after transfer to the experimental environment, and during the season of the year when the rate of metabolism is high (January to May). That it could not be used to predict with any degree of accuracy the oxygen consumption of fishes of varying weight under different conditions, and over a considerable temperature range, such as obtained in the continuous temperature experiments (Wells, 1935), is quite evident. Until we have a series of curves showing weight relations at various temperatures, but otherwise under the same experimental conditions, we are not justified in "correcting" to a constant temperature values obtained over a wide range of temperatures from fishes of even the same species but which differ considerably in weight.

VARIATIONS IN RESPIRATORY METABOLISM DUE TO SEASON AND CONTINUED CONSTANT TEMPERATURE

The plan was to subject one group of fishes to cold water, another to warm water, and to determine from time to time whether any change was taking place in their rates of metabolism. These were to be determined for both groups after transfer to water of the same temperature. First, however, a preliminary experiment was undertaken. Twenty-six fishes were selected, thirteen males and thirteen females, which had been used several times in experiments relative to sex differences in metabolism. For several months these fishes had not been subjected to temperatures below 20°. They were placed in the respiratory chambers in the constant temperature bath at 20°. After 36 hours, during which time several serial determinations were made of their metabolism at 20°, the temperature was lowered to 12°. This required about 6 hours. As soon as the temperature had dropped to 12°, a value of their oxygen metabolism was obtained, the next value being obtained after 12 hours. They were kept at the low temperature 42 hours in all, and during this period twelve determinations of their oxygen metabolism were taken (Table I). When the

fishes were removed from the respiratory chambers they were placed in a tank in which the temperature of the water was kept below 12°.[3] At the end of 60 days their respiratory metabolism was again obtained at 12° (Table I), and the values after the expiration of 24 hours compared with the previous ones. They were found to show an

TABLE I*

OXYGEN CONSUMPTION IN RELATION TO SEX

Hours after reduction to 12°	A		B
	Males	Females	Both sexes
10	.045	.051	.081
12	.059	.055	.063
14	.049	.049	.076
16	.048	.045	.062
18	.046	.046	.083
20	.044	.045	.099
22	.044	.043	
34	.045	.053	Average .077
36	.068	.053	
38	.048	.046	
40	.043	.044	
	Average .048	Average .048	

* A: The consecutive values of the oxygen consumption at 12°, of thirteen males and thirteen females, commencing 10 hours after the temperature had been lowered to 12° and 48 hours after transfer to respiratory chambers. The oxygen consumption is expressed as cc/hour/gram.

B: Six consecutive bi-hourly values at 12°, 60 days after the fishes had been subjected to cold water (10°–12°) and 24 hours after introduction into respiratory chamber.

increase of more than 50 per cent in the oxygen consumption over that obtained when the temperature was first lowered. Elsewhere I have discussed this experiment in connection with a possible group effect in *Fundulus*, and showed that there is no reason to suppose that one exists.

Another significant point is the greater variation in the bi-hourly values, which is indicative of greater muscular activity and suggests in turn that the fishes were becoming acclimatized to the lower temperature. After this experiment they were returned to the cold stock

[3] A cooling unit was provided by which a small stream of sea water could be cooled to 10°. Later, when the temperature of the running sea water in the laboratory fell below 20°, a small heating unit was installed which made it possible to keep the temperature of a small stream of water above 20°.

tank, now at a temperature of approximtely 10°. It was planned that after one more series of values had been taken at the low temperature, the set would be divided into two groups, one to be kept at the low temperature and the other above 20°. Before they could be used again, however, most of the fishes had died from the disease described by Wells and ZoBell (1934). One other set of smaller fishes, which were kept in the same cold tank and had been used once, also succumbed to the disease.

On January 24, 1933, a third set was started. Twenty-seven fishes, which had been kept in a warm tank (20°–24°) since December first, were divided into two groups of fourteen and thirteen. These were placed in the respiratory chambers and the oxygen consumption of each was determined first at 20° and then at 12°. The fourteen fishes were then placed in the cold tank at 10°–12°, and the set of thirteen were returned to the warm water. These two sets at the beginning of the experiment were as nearly of equal size as could be selected. The set of fourteen ("A") had an average weight of 5 grams and the set of thirteen ("B"), 4.8 grams. They were used at various intervals until August 17. In June, however, during the severe epidemic of infectious dermatitis, seven of the group of fourteen kept in the cold tank were lost; hence, the July and August measurements were made on only seven fishes from each temperature group. The average values of all the experiments done with these two sets at the two temperatures 12° and 20° are plotted in Figure 2. In this figure the value of the oxygen consumption of each group of fishes is plotted against time. The average weight of the fishes is indicated at each point on the graph. The fishes kept in cold water are designated group "A," those kept in warm water as group "B." The two curves drawn in broken lines indicate the calculated value of the oxygen consumption of each group of fishes on the basis of an average weight of 5 grams. Data which made possible these calculations were obtained by use of the curve (Fig. 1), which is discussed in detail in the section dealing with the relation of size to metabolism.

Since at present it is impossible to separate the effects on the rate of metabolism due to continued constant temperature from those due to season, I shall first describe those experiments designed to study seasonal effects before attempting a discussion of the results.

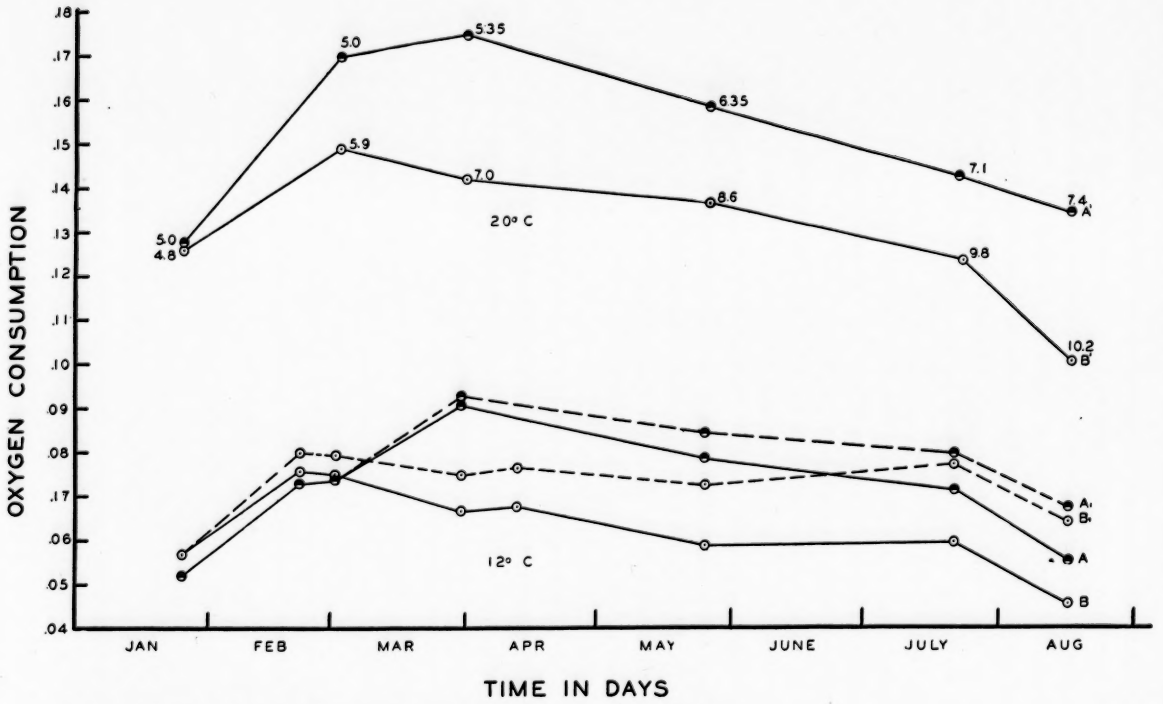

FIG. 2.—Relation of continued constant temperatures to oxygen consumption.

Ordinates represent oxygen consumption in cc. per hour per gram; abscissas, time in months. Curves A and A' represent the oxygen consumption of the "cold-adapted" fishes at 12° and 20° C., respectively. Curves B and B', the oxygen consumption of the "warm-adapted" fishes at 12° and 20° C., respectively. Curve $\overline{A}_1$ represents the calculated values of the oxygen consumption at 12° C. of the "cold-adapted" fishes; B_1, the calculated values of the oxygen consumption at 12° C. of the "warm-adapted" fishes. These values are calculated on the basis of a mean weight of 5 grams. Average weight of fishes in grams is indicated at each point on the 20° curves.

In order to study the relation of season to metabolism it was planned to use several groups of fishes belonging to the same species but of different mean size, and to determine their rates of oxygen consumption at intervals for a year. Small fishes were chosen, since it was known that they would increase considerably in size, particularly in the summer when the water became warm. The values obtained from the four groups throughout the course of a year would, therefore, be representative of nearly the entire weight-range of this species. Since it was planned to conduct all tests at a temperature of 16°, the data could be used to determine the relation of size to metabolism at a constant temperature.

On December 20, 1932, four sets of fishes were selected. The individuals of each set were of equal size and the average weights of each set at the beginning of the experiment were 0.68, 1.34, 1.85, and 2.47 grams respectively. They were placed in battery jars and all subjected to the same feeding and temperature conditions. Four sets of serial determinations of the oxygen consumption of each group of fishes were secured from December 20 until March 23. Unfortunately, at this time so many had become diseased that it was necessary to discontinue the experiments. The values from these four sets, plotted from averages of several determinations, made after a period of 24 hours from time of transfer to the respiratory chambers, and held at a constant temperature of 16°, may be found in Figure 3. The oxygen consumption of each group of fishes is plotted against time, and the mean weights of each group are indicated on the graph. The results of these experiments, together with the results from the constant temperature experiment, will be discussed in the following pages. I shall attempt to show that there are seasonal changes in the rate of oxygen consumption as well as changes induced by subjecting fishes to continued low temperature, and that the effects due to these two factors can be separated.

DISCUSSION OF RESULTS

It seems certain that there is some adaptation to high and low temperature in fishes, such as that described for rabbits by Ogle and Mills (1933). On several occasions I have gradually raised the temperature of the water in which *Fundulus* were kept to 36°–40° without

any apparent ill effects on the fishes. If fishes are returned to cold water (below 16°) within a few hours after the temperature has been

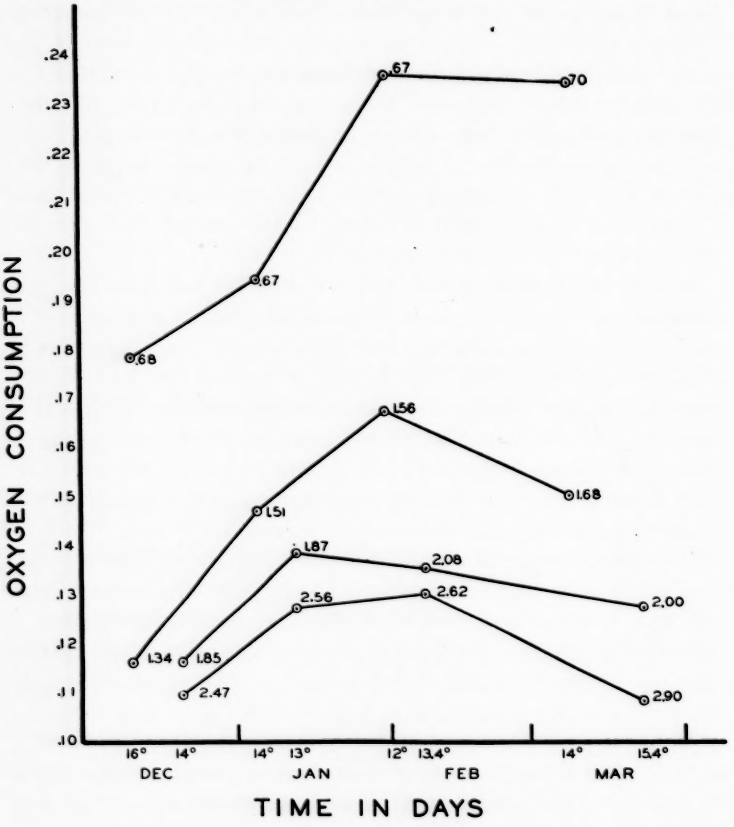

FIG. 3.—Relation of season to oxygen consumption.

The four curves represent the oxygen consumption in cc. per hour per gram of fishes at 15° C. plotted against time, for four groups of fishes. Weights of fishes in grams are indicated at each point on the curve. The temperature of the water from which the fishes were taken is indicated for each experiment on the abscissas.

raised to 36°, their adjustment to the cold water is very rapid, and within a few minutes they are swimming normally. But after a period of 3 days in the warm water, it requires several hours to effect

an adjustment. In no case have I been able to transfer fishes, which have become adjusted to temperatures above 35° for over 8 days, directly to cold water and have them survive. Transferring fishes directly from water below 18° to water above 34° is impossible, as they will invariably be killed in from 5 to 15 minutes. An interesting phenomenon, and one which is indicative of a physiological change in the fish, is an apparent loss of melanin pigment at the high temperature. In appearance, it is similar to that found by Sumner and Wells (1933) with the guppy, *Lebistes reticulatus*, when these fishes were maintained on a white background for long periods of time. This loss of pigment occurs in *Fundulus*, however, in 48 hours or less and is not regained until several weeks after the fishes have been returned to water below 20°.[4]

As yet no experiments have been conducted to determine the rate of respiratory metabolism at these high temperatures,[5] but it is hoped that some can be completed in the near future. For the present, it will be necessary to confine myself to an analysis of the data obtained at the lower temperatures.

Let us consider for a moment two groups of fishes of approximately the same size. What would occur if one group is subjected to the maximum temperature which it would encounter in its normal environment and the other to the minimum temperature? If there is any natural adjustment to seasonal temperature changes one would expect a difference in their rates of metabolism when both groups are subjected to the same temperature. But would keeping the fishes at the two temperature extremes prevent seasonal changes in their metabolism due to other causes? The results of my constant-temperature experiments certainly furnish evidence that, regardless of the temperature to which the fishes are subjected, there are seasonal variations in their rates of metabolism which coincide *approximately* with the variations occurring in fishes subjected to the changing seasonal temperatures (compare the high points in the curves on Fig. 2 with the high points in the curves on Fig. 3). They furnish evidence

[4] It is planned to make an intensive study of this phenomenon in this laboratory within the next year.

[5] Until quite recently, no adequate facilities have been available in this laboratory for heating sufficient quantities of sea water to conduct such an experiment.

that, after continued subjection to low temperatures, fishes show a recovery from the immediate depressing effect of these upon metabolism, that this relative increase is manifest both at high and low temperatures, and may be distinguished over and above the changes due to seasonal variations. They also show that continued subjection of fishes to high temperatures does not prevent seasonal changes in their rates of metabolism. These results indicate that there is a natural rhythm in the physiological functions of fishes that cannot be completely effaced by relatively short periods of reversal of natural conditions.

Let us compare the data in Figure 3, obtained from the fishes kept at the changing seasonal temperature, with those of the two sets of fishes kept at constant temperatures. The values for the former show the highest rate of metabolism occurring in January and February, while the figures for the fishes kept in warm water show the highest rate occurring late in February (Fig. 2). These results are entirely in accord with the seasonal water temperatures recorded at this institution. Thus, the high rates of metabolism occur during the latter part of January and February, the months in which the lowest recorded water temperatures occur.[6] That the period of highest metabolism for the "cold-water" fishes seems to have been shifted over to the end of March may be due to the fact that no values of the metabolism were obtained between March 2 and 31. However, one would expect that the continued low temperature might tend to prolong the period of time in which the fishes maintained the higher rate of metabolism.

In order to separate completely the relative increase in metabolism following subjection to cold water from that due to season, it would be necessary to know the relation between the size of the fishes and the rate of metabolism at any given temperature. As I have shown elsewhere (Wells, 1935), there is a decrease in the absolute relation between size and rate of metabolism with decreasing temperature. Since such is the case, there should be less difference in this rate at 12° than at 16° over the range from 5 to 10 grams; hence, to apply

[6] From the 18-year averages of the water temperatures obtained at this station the following values were taken: December, 14.81°; January, 13.98°; February, 13.95°; March, 14.69°. The highest temperature occurs in August.

a correction from the 16° curve (Fig. 1) to values obtained at 12° is quite legitimate in so far as weight-metabolism relations are concerned. Therefore, if we calculate what percentage of the difference between the cold-adapted and warm-adapted fishes (*A* and *B*, Fig. 2) is due to increase in weight, we should get an approximate measure of the effect due to the cold temperature above that due to season. Accordingly, I have calculated what the value of the metabolism would have been if the fishes had remained at the original weight of 5 grams, and have plotted these values and connected them for each series of fishes by broken lines (Fig. 2). It may be seen from these graphs that the seasonal increase at 12° during the first 27 days was approximately the same for the "warm-water" fishes as for the "cold-water" ones, and that there was perhaps a slight retarding effect on the fishes kept in cold water. Since these fishes not only gained no weight, but lost slightly, it is probable that some time is required for them to become adapted to the cold temperature. In regard to this initial increase in oxygen consumption by the cold-adapted fishes, which is 42 per cent, it may be recalled that in the first experiment with the thirteen males and thirteen females (Table I) the increase after 60 days was 54 per cent. In this case, however, the fishes increased in average weight from 5.7 to 6.0 grams while in the cold water. One further point that must be considered is the difference in the history of the two groups prior to being placed in the cold water. The first group of twenty-six males and females had been subjected to the changing temperatures of the laboratory sea-water supply which had not been above 20° at any time, while the second group of fourteen had been in water at a temperature from 24° to 28° for 2 months. Since the fishes of this latter group were forced to adjust themselves to a greater temperature change, it would probably require a longer period of time.

As soon as the "cold" fishes became adjusted to the lower temperature, indicated by a resumption of growth, there was a marked increase in their rate of metabolism over that of the "warm" fishes, at 12°, so that by the end of March there was a 20.4 per cent increase over the "warm" ones, when the values of the metabolism are calculated on the basis of an average weight of 5 grams. In the latter part of May this difference had fallen to 16.5 per cent. The differences in

July and August are not significant, but it should be borne in mind that by July one-half of the cold-tank fishes had died of disease and within 10 days after the August experiment, four of the remaining seven had died. ZoBell and Wells (1934) have shown that these fishes are more susceptible to the disease when the temperature of the water is 20°–25° and, since the fishes in the warm tank were not affected, it indicates that those in the cold tank may have been in a weakened condition due to the prolonged low temperature and, hence, were susceptible to disease.

It is known that even in man there are adjustments in the rate of metabolism to cold and warm climates. Heinbecker (1928) found the basal metabolism of Eskimos to be 33 per cent higher than that of inhabitants of temperate zones. Hafkisbring and Collett (1924) found their own basal metabolism to be about 5 per cent higher in cold weather (3°–10°) than in warm weather (20°–27°). Collett found hers to be 8.8 per cent higher at temperatures from 0° to 10° than from 20° to 30°. Turner and Aboushadid (1930) quote de Almeida as stating that he found the basal metabolism of Brazilian whites to be 24 per cent below the Harris and Benedict standard. Gessler[7] found his own metabolism to be about 7 per cent above the average in winter and 6 per cent below in summer. DuBois, in reviewing the seasonal effect on metabolism, states: "If these variations apply to many subjects it is of great importance since it would oblige us to use different standards in winter and in summer." He further calls attention to the fact that most of the high values given in Table 17 of his book, *Basal metabolism*, were obtained during the cold months, and most of the low values in the warm months. While no parallel can be drawn between the metabolism of homoiotherms and poikilotherms owing to the almost complete dependence of the latter on environmental temperatures, it is significant that even in man there is an indication that the temperature of the environment influences basal metabolism.

It must be concluded from the experiments described that the rate of metabolism of *Fundulus purvipinnis* at any given temperature is a function of the temperature to which the fishes have been

[7] Gessler is quoted by Dubois (1927) in his book, *Basal metabolism*.

subjected prior to the experiment and probably a function of the duration of that time. The problem is far from being solved. In any work, however, dealing with the temperature-metabolism relation of cold-blooded vertebrates the question of the duration of time the animals are subjected to any given temperature must be considered before the results can be fairly evaluated.

SUMMARY AND CONCLUSIONS

The relation of size to the rate of metabolism of *Fundulus* at a temperature of 16° has been determined. It is emphasized, however, that these same relations may not hold at other temperatures.

Fishes subjected to high and low constant temperatures for a period of several months show, nevertheless, a seasonal change in their rates of metabolism coincident with the seasonal changes occurring in the rate of metabolism of fishes that were maintained at the changing seasonal temperatures. The fishes which had been kept in cold water had a higher rate of metabolism, when tested both at 12° and 20°, than did the fishes maintained in warm water. From these results it may be concluded that the rate of metabolism of fishes at any given temperature is dependent upon the temperature of the water to which they have been acclimatized.

The variation of respiratory metabolism of *Fundulus* with season seems to be definitely established. It was found to occur with fishes maintained at high and low constant temperatures as well as with fishes kept at the changing seasonal temperatures. Records of metabolism from December to August indicate a high rate during February and March and a low rate during July and August. In any comparative studies of the respiration of fishes this factor of seasonal change must be considered.

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EXPERIMENTS ON THE CASE-BUILDING OF THE CADDIS-FLY LARVA, NEURONIA POSTICA WALKER

(Three figures and two plates)

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THE building activities of caddis-fly larvae offer an unusually favorable opportunity for the study of insect behavior under various experimental conditions. Removed from their portable cases, they construct new ones within a comparatively short time. The process of construction of the case can thus be followed in detail. Moreover, the animal itself, in building a new case, lays down a permanent record of its activities, which can be preserved for further study and comparison.

The architecture of the natural case or the material used in its construction, or both, are in many instances characteristic for a given species. Certain materials are selected from those present in the pond or stream, prepared, and fastened together by a secretion of the spinning glands according to a definite pattern. Under normal conditions, all individuals belonging to the same species select the same materials and arrange these in essentially the same way. The whole series of activities thus appears to follow a definite pattern that is prescribed by the hereditary constitution of the species. It is an example of that type of behavior which may be designated as 'instinctive' until the analysis of such behavior will have led to a better understanding and to the adoption of a more suitable term.

Many of the numerous papers dealing with the habits and life-histories of various species of caddis-fly larvae contain observations on their behavior under laboratory conditions. A few investigators only, however, undertook a methodical experimental study of the case-building activities of the larvae (Ostwald, 1899, 1901; Marshall and Vorhies, 1905; Bierens de Haan, 1922; Uhlmann, 1923, 1924, 1932; Dembowski, 1924, 1933; Gorter, 1928, 1931). Gorter (1931)

has given an extensive and rather complete review of the literature on this subject. The following results of this experimental work are particularly significant:

1. A species that builds in nature with only one kind of material (e.g., with fragments of dead leaves or sand grains) may, in the absence of this material, use a variety of others. This 'building-range'—*Pluripotenz* (Uhlmann), *Instinktbreite* (Bierens de Haan), *Wahlbreite* (Gorter, Dembowski)—appears to be considerable in most species; while others, such as some mineral builders, exhibit a much more limited power of handling strange materials successfully.

2. The new cases may be built on the normal pattern, as far as the nature of the new material will permit, or they may be fashioned in a different style that is better adapted to the properties of this material.

3. If given the 'choice' between two or more different materials, the larvae frequently show a distinct 'preference' for one of these, using it more abundantly or exclusively in the construction of the new case. All the materials that are tested in such experiments may thus be arranged in a definite sequence, the one at the top of the list being always 'preferred' to any of the others (cf. the extensive lists given by Gorter, 1931). The 'preference' (*Vorliebe* or *Prävalenz* of the German-speaking authors) was discovered by Bierens de Haan (1922) in experiments with *Limnophilus marmoratus*, and investigated further by Uhlmann (1923, 1924, 1932), Dembowski (1924, 1933), and Gorter (1928, 1931).

So far, the larvae of the family Phryganeidae have hardly been tested in experiments, although they construct unusually regular cases. Ostwald (1899) investigated the building range of larvae of *Phryganea striata*, and Uhlmann (1924) mentions some 'preference' experiments on *P. grandis*. Recently, Teyrovsky (1934) described briefly the repair of damaged cases of *Neuronia ruficrus*. No experiments have been made, as far as we know, on the case-building of American species of *Neuronia*, phryganeids which fashion beautifully regular cases composed of six-sided rings of leaf fragments. The striking pattern of these cases is particularly valuable in experiments designed to test the adaptability of the building activities of

the larvae to strange building materials. Any change in the well-characterized sequence of the building activities as well as in the pattern of the completed case would be easily detectable.

The larvae of *Neuronia postica* Walker can be found throughout the winter in sluggish streams, where they crawl among the dead leaves at the bottom or climb on the water-cress. Preliminary experiments proved the *Neuronia* larvae to be very active builders that replace the original case quickly and several times in succession, if the new cases are removed. Furthermore, they build during the day as well as at night, and can therefore be watched without difficulty.

In addition to the ordinary tests of the building range and 'preference' of the larvae, experiments were planned to investigate two particular problems: (1) Is the normal 'preference' of the larvae influenced by variations in the relative abundance and in the size of the materials given? (2) Are the larvae able to build normal cases following the amputation of one pair of legs?

OBSERVATIONS

1. MORPHOLOGY OF THE LARVA

A full-grown larva, removed from its case, is shown in Figure 4, Plate I. The soft-skinned, green-colored abdomen carries numerous filamentous gills. The last segment bears two small sharply curved and pointed dragging hooks, which are normally anchored in the lining of the case near its posterior end. (For a detailed description of the larva cf. Vorhies, 1909, and Lloyd, 1921.)

If the body wall is split along the mid-dorsal line from anus to head and the cut edges are pinned down under physiological salt solution, we obtain the view represented in Figure 5. The tracheae are still filled with air and are conspicuous by their silvery appearance. The intestine is filled with food—particles of dead leaves—and clearly divided into three sections: a large crop, separated by a constriction from the mid-gut, which is characterized as a region of higher physiological activity by the more numerous tracheae, and a short hind-gut. The whole body cavity is lined with leaflike fat-bodies of a greenish color.

The spinning glands are not visible in the photograph because they are translucent in the fresh dissection. If the salt solution in

the dish is replaced by a mixture of alcohol and acetic acid, the fixing fluid coagulates almost instantly the secretion in the glands and the protoplasm of the secreting cells. The posterior, convoluted portion of the two glands becomes clearly visible on both sides of, and posterior to, the mid-gut (Fig. 6). In this region, but more laterally, the small gonads also stand out more clearly from the surrounding fat tissue as white bodies.

The general shape and size of the spinning glands are shown in Figure 7. As the two tubes enter the head, they taper to very fine ducts that run parallel near the ventral mid-line until they reach the lower lip, where they open at the tip (Fig. 8). The joint portion of the ducts ('silk press') must be much shorter than in *Limnophilus* (Gilson, 1894) and *Platyphylax* (Vorhies, 1904). Feulgen's reaction brings out beautifully the branching nuclei of the secreting cells in whole mounts of the spinning glands (Figs. 9 and 10).

Fig. 8 also shows the mandibles which are used for cutting the leaf segments that serve as building material, and the striking differences between the first and second pairs of legs. The former are modified into powerful grasping organs, while the mesothoracic legs are slender and resemble closely the metathoracic legs.

2. THE NORMAL CASE AS BUILT IN NATURE

The case of *Neuronia* (Fig. 12, Plate II) is a slightly curved or straight tube, wide open at both ends. The diameter may be the same all along the tube or increase slightly toward the anterior end. It is usually six-sided in cross-section and consists of from three to five rings of building units which are placed end to end. There is very little overlapping of the rectangular leaf segments, which are cut and fitted together with remarkable regularity. All natural cases collected around Princeton were made of segments of dead leaves or grass blades, or of a mixture of both.

Adjoining rings contrast frequently in color, as they are often made of different materials (Fig. 12). Each ring is usually uniform in its material, although segments of leaves and grass blades may sometimes be used in the construction of the same ring (anterior ring of the smaller case in Fig. 12). This condition agrees with the observation made in the laboratory that the larva, once it has begun

to add a new ring at the anterior end of the case, completes this addition rather rapidly, often with segments of the same leaf; on the other hand, the larva may pause for several days, or even weeks, before it adds another ring in front.

The case of *Neuronia* (Fig. 11*C*) is so typical that it can be distinguished at the first glance from that of *Phryganea* (Fig. 11*D*) and the various limnophilid cases that occur in the same locality (Fig. 11*A*, *B*, *E*, *F*).

3. THE CONSTRUCTION OF NEW CASES WITH NORMAL MATERIAL

Most experiments were carried out from November, 1932, to April, 1933. Siltala (1907) and Wesenberg-Lund (1911, 1913) state that the larvae of European Phryganidae grow rapidly during the summer and early fall and reach the last larval stage in October. In this stage they pass the winter months. It is therefore probable that the majority of the larvae used in our experiments were fully grown or nearly so. Differences in the building behavior of young and old larvae, which have been observed in many other species and which may also be present in *Neuronia*, need, therefore, not be considered here. The animals were kept, at ordinary room temperature, in individual finger bowls, providing ample space for their activities.

Immediately after removal from their old cases, the larvae became extremely active, crawling restlessly about the bottom of the dish and pausing to investigate whatever materials were present. Whenever it was possible, the larvae soon burrowed out of sight under such materials, and from this position they would begin the construction of a new case.

The larvae invariably required some more or less firm point of attachment from which to start constructing their new cases. (1) If the materials provided were small (e.g., dead leaves cut into small segments), these would be gathered and fastened together with silk to form a rough pile. The new sheath was constructed with other fragments but remained attached to this pile until, eventually, the larva turned around inside the sheath and cut it off. Figure 13 shows part of the original pile of leaf fragments still fastened to the posterior end of a new case in an advanced stage of construction. (2) In other experiments the larvae were given a single large leaf. Almost

immediately they crawled under this leaf and soon began to cut out pieces from the edge. Within a few hours a new case fastened to the leaf would be completed, to be ultimately cut off from this support. (3) If the quantity of building material was extremely limited, so as barely to suffice for the construction of the case, the larvae sometimes started the building process by gluing a few pieces to the glass of the dish.

The cutting of a building unit may be preceded by an 'examination' of the original piece of material. If it is a blade of grass, the larva, grasping it between its prothoracic and mesothoracic legs, passes up and down the blade; then, by means of the mandibles, cuts it across at some point which does not always happen to be near one end. If the piece retained in its grasp is still too long, another cut is made to provide a building unit of suitable length. If the larva is building with leaf, it usually passes back and forth along the edge before cutting out a piece. The whole process resembles the preliminary 'sizing-up' of a piece of material by a carpenter. It may be reduced to a minimum in the early stages of the construction of a new case, when the larva works rapidly and appears less exacting as to the regularity of the building units.

Once the building unit has been cut, the larva next secretes some threads of silk over the entire surface that is to form part of the inner wall of the case. Then one end of the piece is brought up against the anterior margin of the case and is there fastened by strands of silk. Both during the cutting of the piece and while it is being affixed to the case, the larva, by means of its prothoracic and mesothoracic legs, shows an efficient handling of the material. With these two pairs of legs, it holds onto the material while it is being cut, turns it around, and finally affixes it to the case. The metathoracic legs are apparently of less importance in the building process.

In the later stages of the building process, the larvae often withdraw into the already completed portion of the case, where they reinforce the silken lining until it forms a tough membrane.

4. PRELIMINARY AND FINAL CASES

Many observers, working with different species of caddis-fly larvae, have recorded that the first portion of a new case built of

vegetable material is less regular than the later additions. The building materials are less carefully selected and trimmed and less regularly fitted together, so that this portion of the case may not exhibit at all the typical features of the normal case. Only when the case has reached a sufficient length to give protection will the animal begin to work more carefully and to build on, at the anterior end, a case with the specific characteristics. Finally, after several days or even weeks, the larva will cut off the irregular portion, which is thus characterized as a provisional protective sheath that is replaced by a less fragile and less bulky construction.

The following points of contrast were observed between the provisional and final portions of new cases built by *Neuronia* (see Figs. 13–15):

1. The building units at first vary considerably in size and in shape, while later on they become more uniformly oblong.

2. The units may be haphazardly placed with regard to the axis of the case, so that in the early structure there may be projecting parts and occasionally gaps.

3. The characteristic ring arrangement of the units is absent in the early building stages.

4. In cross-section, the first portion of a sheath is usually roughly circular or polygonal, while later on it becomes definitely hexagonal.

5. While in the early stages of construction the larvae build with whatever materials are convenient, later on they confine themselves to the use of a single 'preferred' material. This point will be discussed more fully in the section on 'preference.'

The main cause of all these differences between the preliminary and final cases seems to be the difference in the speed of construction. The early portions of the sheath are constructed rapidly, at the rate of 2–3 cm. in 4 hours. Well-defined rings are added, later on, to the anterior end much more slowly.

The transition from the first to the second type of construction is, of course, not abrupt. Although the early part of the case does not show the uniform ring arrangement, the larvae, nevertheless, place the units side by side, with their long axes roughly parallel; and, when one of the units extends somewhat beyond the others, they never add another piece to it until the entire anterior margin has

been brought up to or beyond it. When the case has reached a sufficient length, the larvae were observed to cut off projecting parts at the anterior, as well as at the posterior, margin. Thus, the anterior margin becomes more or less uniform. There follows a pause in the building activity, and then other units are added. The projecting pieces are likewise cut off, and we have the first well-constructed ring of the final case (Fig. 15).

That the larvae, once adequately inclosed in a case, tend to cut off projecting parts at the anterior or posterior margins is further evidenced by experiments in which the cases were damaged by cutting out pieces from either margin. At the posterior margin, the portion projecting beyond this gap was invariably cut off. At the anterior margin, when the gap was large and the case of sufficient length, the projecting part was likewise cut off; if not, the gap was filled in, and, if the added piece in turn extended beyond the others, it was trimmed down.

5. BUILDING RANGE

In a series of experiments a variety of materials was supplied to *Neuronia* larvae that had been removed from their cases to test their building range.

a) Perfectly typical cases were built out of green leaves, cabbage leaves (Fig. 16 *B*), onion skin, and cellophane. All these materials, including cellophane, were cut by the larvae in normal fashion; and the building units were united first to a rough preliminary and then to a more regular final case. The perfectly transparent cellophane cases were particularly well built. Fig. 17 shows how neatly the oblong building units were fitted together. The picture was taken in transmitted light, to show the fine threads of the silken lining which form an irregular meshwork. The edges of the units are not fastened together by neat seams, as are the sand grains in the cases of *Molanna* (Dembowski, 1933), but are united by a heavy, irregular band of silk of varying width. It may be seen in the picture that the meshwork of fine threads lining the whole building units becomes more dense toward the right, i.e., the posterior portion of the case.

b) Ordinary white paper was cut by the larvae only with difficulty. If the paper was supplied in narrow strips, typical cases were

constructed, the strips being affixed whole or first cut down to regular building unit size (Fig. 16 *D*). Small pine-shavings were never cut by the larvae; but cases were readily built, at first with pieces of varying size. At the end of 1 or 2 days, such cases even exhibited a ring arrangement in the anterior portion, because only smaller shavings of roughly uniform size were used in the later stages of construction (Fig. 16 *A*).

c) The larvae were even able to cut rather heavy tin foil. But they seemed to have difficulties in fastening the roughly oblong pieces together, and the resulting case remained crude and unstable.

d) Very crude cases were built out of oatmeal and barley.

e) If given twigs, pine needles, or pieces of wire, the larvae would fasten a few together but would not go any farther in the building process. With pieces of string, the larvae did not show the slightest tendency to build, but tried to get what shelter they could by simply crawling under them where they were thickest.

f) The construction of cases out of sand grains or small pebbles deserves special consideration. When only sand or fine gravel was furnished, the larvae first collected and fastened together a pile of grains as a point of support. One larva, placed in a finger bowl with sand and some fairly large pebbles, joined a number of these together to serve as a support. Although the larvae were able to construct compact, cylindrical cases out of sand, they did not use it as readily as other materials. Of fifteen larvae which were tested at various times with sand alone, seven never built a case. The remaining eight did not begin with the construction of a case until the third day or later, and the building activity ceased after 2 or 3 days, when the case had reached a length of from 15 to 20 mm.

However, if, in addition to sand, pine needles or small twigs were given, the building process took a different turn. Where the needles or twigs were fairly thick, the larvae fastened some of them together with strands of silk, without attempting to cut them into smaller units. Usually two or more of these needles or twigs were parallel, and others lying across them held them in place. Using this structure as a framework, they began to fasten grains of sand to the underside of the parallel needles, hanging-basket fashion, and continued with this process until the sides and the floor of a crude case

of adequate length were formed. The upper length of the case, between the parallel needles, contained very little sand. During the next 24 hours, the larvae went over almost the entire length of the case, filling in the gaps between the parallel needles (Fig. 1) and

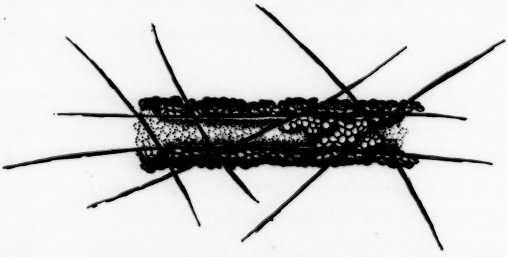

FIG. 1.—Construction of case out of pine needles and sand, 48 hours after beginning of experiment (larva No. 7, Table II). Case as seen from above, showing the incomplete upper portion. Pine needles fastened together, with the incompletely roofed sand structure attached to, and hanging down from, needle framework. (Early stage III.)

adding comparatively little sand at the anterior margin. Sometimes the case was extended by the addition of sand, not only to the anterior, but also to the posterior, margin. As the case became more compact, it was observed that some of the needles had been discarded

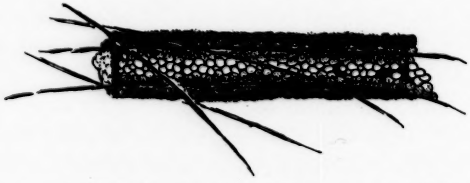

FIG. 2.—Same structure 30 hours later. Gap between two parallel needles filled with sand; some needles have been discarded, and the remaining ones are loosely attached. (Stage V.)

and that the remaining ones, although in approximately the same position relative to the case, were very loosely attached (Fig. 2). Apparently the larva, inserting sand at the original points of attachment of the needles, gradually severed the connecting strands of silk until the needles became entirely detached from the sand case (Fig. 3).

Eight larvae were subjected to this experiment with pine needles

and sand. The building process was very much the same with all of them, and consisted of the following series of stages of construction:

I. Needles fastened together
II. Sand structure hanging down from needle framework
III. Sand filling interstices between needle framework above
IV. Sand case more compact, needles only loosely attached
V. Few needles still attached
VI. Case free of needles

The condensed record of this experiment is given in Table I to illustrate the individual variations in the speed of construction.

While, with pine needles or twigs alone, the larvae never build a case and are slow in constructing a sheath with sand alone, if they attempt it at all, a combination of the two types of material apparently enables them to build cases of normal length much more readily. However, the usual method of building is profoundly altered to suit the unusual conditions: (1) The cluster of needles or twigs resembles the pile which is collected by the larvae as a point of attachment for the case, if the building materials provided are small; but it is here used as a scaffolding supporting the whole extent of the case and is gradually disconnected from the sand structure as the latter becomes more compact. (2) The material is not added in successive rings to the anterior margin of a case of insufficient length; but a loose, incomplete structure of nearly full length is built, supported by the scaffolding, and reinforced later on over its entire length.

FIG. 3.—Finished sand case 18 hours later. All needles removed. (Stage VI.)

The larvae of *Neuronia postica* thus show a surprising plasticity of their building behavior which enables them to attain the typical end-result, a cylindrical, compact case, by a temporary combination of two widely different building materials.

6. 'PREFERENCE' EXPERIMENTS

In all experiments which were devised to test the 'preference' of the larvae for certain building materials, only two kinds of material were given each time. No attempt was made to try a large variety

of materials and to determine their relative position in a 'preference list' corresponding to those given by Gorter (1931) for *Limnophilus*. The aim of these experiments was to determine whether or not variations in the size of the pieces of material furnished or in their relative abundance would influence the choice of the larvae.

Under natural conditions the larvae of *Neuronia postica* build with segments of dead leaves or of grass blades. In the first group of experiments, dead leaves were tested against grass, pine shavings, and

TABLE I

CONSTRUCTION OF CASES WITH PINE NEEDLES AND SAND

Eight larvae were removed from their cases and placed in finger bowls containing a liberal quantity of long pine needles and sand.

The Roman numerals in the columns designate the six successive building stages as defined in the text.

Columns left blank indicate no building activity observed.

Experiment begun December 28, 1932, 9:30 A.M.

Larva No.	12/29/32 5:30 P.M.	12/30/32 11:00 A.M.	12/31/32 11:15 A.M.	1/1/33 9:00 A.M.	1/3/33 2:45 P.M.	1/6/33 11:30 A.M.	1/7/33 2:00 P.M.	1/9/33 2:15 P.M.
1	II	III	IV	V	VI			
2		I	II	III	V	no further change		
3		II	III		V	VI		
4			I	II	III	V		VI
5			I	II	III	V		VI
6		II		IV	V		VI	
7	II	III	IV	VI				
8	II	III		IV	V	VI		

cellophane (Table II, Expts. I–IV). The results show a marked 'preference' of the animals for leaves, although they are able to construct normal cases with the three other materials if dead leaves are not available (see the preceding section). In the construction of the final case (last column of Table II), the 'preference' for leaves is always clearly exhibited, whether the leaf be given whole or cut up into small pieces of approximate building-unit size (Expts. III and IV). During the early stages of construction, however, when the larvae build rapidly, they always 'prefer' material which can be used without cutting (small pine shavings, small pieces of cellophane) or can be easily trimmed down to a convenient size (grass blades).

All larvae used in the same experiment behaved in essentially the same way, as is evidenced by the complete record of Expt. II (Table III). While individual differences existed in the speed with which a case of leaf segments was completed, all five larvae had abandoned the use of shavings on the morning of the second day.

TABLE II

SUMMARY OF 'PREFERENCE' EXPERIMENTS

In each experiment two different materials (A and B) were given to a number of larvae which had been removed from their cases and placed in individual finger bowls.

Expt. No.	No. of Larvae	Material A	Material B	Proportion of A and B	Material Used during First Hours	Material Used Later On
I.....	5	leaf 5 cm. sq.	grass 5 cm. long	.....	grass	leaf
II....	5	leaf, whole	pine shavings, small	.....	shavings	leaf
III....	5	leaf, small pieces	pine shavings, small	1:1	leaf and shavings, 1:1	leaf and few shavings
IV....	3	leaf, whole	cellophane, small pieces	1:1	cellophane	leaf
V.....	3	leaf 5 cm. sq.	paper, small pieces	.....	paper	leaf
VI....	6	leaf, small pieces	paper 5 cm. sq.	.....	leaf	leaf
VII...	5	leaf, small pieces	paper, small pieces	1:1	leaf and paper 1:1	leaf and few pieces paper
VIII..	8	leaf, small pieces	paper, small pieces	1:2	leaf and paper 4:5	leaf and paper 2:1
IX....	8	leaf, small pieces	paper, small pieces	1:3	leaf and paper 1:3	leaf and paper 1:1
X.....	10	cellophane, small pieces	paper, small pieces	1:1	paper and some cellophane	cellophane
XI....	10	cellophane, 7.5 cm. sq.	paper, small pieces	.....	paper	cellophane (8 larvae)

The second group of experiments (Table II, Expts. V–IX), including various leaf-paper tests, showed the same 'preference' for leaf material in the later building stages, whatever the size in which the pieces of the two materials were furnished (Expts. V–VII; Fig. 18*C*). If, however, the proportion of small leaf and paper segments was altered in favor of the latter (Expts. VIII and IX), the 'prefer-

ence' for leaf material did not express itself completely. Even at the close of the second week, pieces of paper were still used (Fig. 18*A*); but the number of leaf segments increased considerably during the later stages of construction, indicating that the larvae selected more leaf segments from the mixture which still contained paper in excess.

When cellophane and paper were furnished, both in small pieces and in equal quantities (Expt. X, Table II), the larvae used more paper than cellophane in the construction of the crude preliminary case, but showed a definite 'preference' for cellophane later on.

TABLE III

'PREFERENCE' EXPERIMENT II: LEAF AND SHAVINGS

Five *Neuronia* larvae were removed from their cases and placed in finger bowls containing one whole dead maple leaf and a considerable quantity of small pine shavings. Experiment begun January 7, 1933, 1:45 P.M.

Larva No.	Jan. 7 4:30 P.M.	Jan. 8 10:30 A.M.	Jan. 8 3:00 P.M.	Jan. 8 5:00 P.M.	Jan. 8 7:30 P.M.
1.....	crude cluster of shavings	case all leaf	case all leaf	case all leaf	case all leaf
2.....	crude cluster of shavings	anterior 2/3 of leaf	more leaf at anterior	case all leaf	case all leaf
3.....	crude cluster of shavings	anterior 1/3 of leaf	case all leaf	case all leaf	case all leaf
4.....	crude cluster of shavings	anterior 2/3 of leaf	no change	no change	case all leaf
5.....	crude cluster of shavings	anterior 2/3 of leaf	case all leaf	case all leaf	case all leaf

Twelve days after the beginning of the experiment, four cases were made entirely of cellophane, while the remaining six showed the exclusive use of this material in the anterior portion (Figs. 18*D* and *E*).

This 'preference' became manifest even if the larvae were forced to cut their own building units out of a sheet of cellophane 7.5 cm. square. Eight out of ten larvae began to cut and use this material between the second and eighth day of the experiment (Table IV).

The 'preference' for cellophane is not as strongly marked as that for leaves, but nevertheless is clearly present. It is probably determined by the mechanical properties of this material, which is at the same time light and firm, while paper tends to become soft after a prolonged exposure to water. Further experiments will be necessary

to decide whether dead leaves are also 'preferred' because of their texture and other mechanical properties alone, or whether chemical factors are involved as well.

TABLE IV

'PREFERENCE' EXPERIMENT XI: CELLOPHANE AND PAPER

Ten *Neuronia* larvae were removed from their cases and given small pieces of paper (approximately the size of normal building units) together with a single piece of cellophane 7.5 cm. square.

Experiment begun March 21, 1933, 10:45 A.M.

Larva No.	Mar. 21 3:00 P.M.	Mar. 22 10:50 A.M.	Mar. 22 3:30 P.M.	Mar. 25 11:00 A.M.	Mar. 29 11:00 A.M.
1.....	crude paper case	case improved, all paper	2 pieces of cellophane in anterior ring	anterior ring cellophane	anterior 2/3 cellophane
2.....	crude paper case	case improved, all paper	2 pieces of cellophane in anterior ring	anterior ring cellophane	anterior 2/3 cellophane
3.....	crude paper case	case improved, all paper	no cellophane	no cellophane	*all cellophane*
4.....	crude paper case	case improved, all paper	no cellophane	1 piece cellophane at anterior	anterior ring, all cellophane
5.....	crude paper case	case improved, all paper	no cellophane	no cellophane	no cellophane
6.....	crude paper case	case improved, all paper	no cellophane	no cellophane	anterior 2/3 cellophane
7.....	crude paper case	case improved, all paper	no cellophane	no cellophane	no cellophane
8.....	crude paper case	case improved, all paper	no cellophane	anterior ring cellophane	anterior ring cellophane
9.....	crude paper case	case improved, all paper	no cellophane	no cellophane	4 pieces of cellophane in anterior ring
10.....	crude paper case	case improved, all paper	4 pieces of cellophane in anterior ring	paper added	*all cellophane*

7. CONSTRUCTION OF CASES AFTER AMPUTATION OF ONE PAIR OF LEGS

Eight *Neuronia* larvae were anaesthetized in a 2 per cent solution of ether in tap water. Both prothoracic legs were cut off as close to

the body as possible. Deprived of their cases, the larvae were placed in finger bowls with several dead leaves. Three died, but the remaining five built fairly compact cases within 18–20 hours after the operation, i.e., approximately within the normal time. However, the building units were more crudely cut and less regularly arranged than in the cases built by control larvae that had been anaesthetized for the same period but not operated upon. Even at the anterior end of 8-day-old cases very few of the building units were at all rectangular in shape, the edges of most of them being more or less curved (Fig. 19). Nevertheless, a crude ring-arrangement was noticeable at the anterior end of some of the cases.

When both mesothoracic legs were cut off, four out of ten larvae survived the operation and built cases. As a whole, the cases were crude and the building process was slow. The building units varied more widely in size and were more irregular in shape than was observed in the experiments involving amputation of the prothoracic legs, although one larva constructed a fairly compact case with roughly oblong pieces at the anterior end.

The experiments seem to indicate that the loss of the mesothoracic legs interferes more seriously with the building activities of the larva than the absence of the first pair. In either case the larva is no longer able to cut rectangular building units of uniform size, possibly because the two pairs of legs are used in some way to measure the dimensions of these units, as has been suggested by Bierens de Haan (1922) for the prothoracic legs of *Limnophilus marmoratus*.

This observation is, however, less important than the fact that the larvae are at all able to construct fairly normal cases after the amputation of one of the first two pairs of legs. The observations of Murphy (1919) on *Brachycentrus nigrisoma*, of Bierens de Haan (1922) on *Limnophilus marmoratus*, and of Gorter (1931) on several species of *Limnophilus* have shown that in these species each pair of legs performs certain functions in the building process. The normal *Neuronia* larva uses both the prothoracic and mesothoracic legs constantly during the cutting of the building units (see section 3). Although our observations have not revealed, so far, a striking division of labor between these two pairs of legs, the morphological

differences (Fig. 8) strongly suggest that they do not perform exactly the same duties.

Whatever may be the degree of specialization of the pro- and mesothoracic legs of *Neuronia*, the larva, after removal of one of these pairs, cannot use the remaining one in exactly the same way as before. Yet the animal is able to adjust its movements to the strange conditions and to build a fairly compact, and sometimes even typical, case. The plasticity of the behavior of the operated animals corresponds closely to that of the crabs, spiders, and beetles in the experiments of Bethe (1930) and Bethe and Woitas (1930). In these animals, following the amputation of certain legs, the movements of the remaining ones were co-ordinated in a different way so as to keep the locomotion as steady and as normal as possible. In *Neuronia* also, a rapid adjustment seems to take place in the co-ordination of the remaining legs which leads to orderly building activities. The final result of these activities, the case, is as close to normal as is possible under the abnormal conditions.

Further amputation experiments on *Neuronia* larvae are planned, which may give more accurate information regarding the changes in the co-ordination of the legs that take place.

III. DISCUSSION

Every observer of the building activities of caddis-fly larvae under experimental conditions must be impressed with the adaptability of their behavior. Two of the experiments reported in this paper demonstrate this point particularly well: the building of cases with pine needles and sand, and their construction after the amputation of one pair of legs. In the first experiment, the larvae are provided with two strange materials, neither of which is used readily without the other. In the presence of both, the pine needles are used merely as a scaffolding to facilitate the construction of a sand case (p. 345, Figs. 1–3). The whole building process is quite different from the normal procedure, but leads to the production of a case which is as typical as the strange building material will permit. In the second experiment, the larvae are given an abundance of dead leaves, their 'favorite' building material; but they are deprived of two important

tools that are used constantly in the normal building process. Again the animals adapt their behavior to the entirely new situation: by means of the tools that are left, they promptly begin to construct a case that will be as similar to the normal sheath as can be expected under the conditions of the experiment.

Dembowski (1933), working with the larvae of *Molanna*, a mineral builder, emphasizes also the plasticity of their behavior and, more emphatically, the wide range of individual variation. On the basis of his observations he disputes the correctness of the term "case-building instinct" as applied to caddis-fly larvae. He attempts to show that the definition of instinct as given by Ziegler does not agree with his findings on *Molanna*, and arrives at the conclusion that the term should be discarded altogether. Dembowski is of the opinion that every activity of an animal will be found to be essentially plastic as soon as our methods of investigation will be adequate.

There is little doubt that the normal building activity of *Neuronia* answers perfectly to Ziegler's definition of an instinct. (1) The ability and the impulse to build a case of a certain type are inherited and common to all individuals of the species. (2) No learning by experience is necessary; although the larvae may possibly become more efficient in handling the material as they grow older, they build typical *Neuronia* cases from very early stages on. (3) The normal building behavior is adapted to the conditions of the normal environment.

Individual variations may be expected of any hereditary character and may be caused by variations in the hereditary constitution of the individuals or by environmental factors, or by both. They may become more obvious under the abnormal conditions of an experiment. Differences do exist between the reactions of individual *Neuronia* larvae in our experiments, but they are unimportant compared with the general tendency of all larvae to react in the same or, at least, in a similar way in unusual situations (Tables I, III, IV). On the other hand, it is a well-established fact, supported by numerous observations, that an instinct which appears rigid under normal conditions may prove to be more or less plastic and adjustable when unprecedented situations arise.

Every action of an animal is a function of both constant and variable factors, as has been stated by Alverdes (1925). The constant, conservative element is preponderant in instinctive actions; the variable, originative element predominates wherever we reach the level of intelligence. This duality appears to be nothing else than an expression of the general rule that both kinds of factors, conservative and spontaneous or indeterminate, are present in any natural occurrence, in the living world as well as in the non-living (Lillie, 1934). "The essential peculiarity of vital organization (as contrasted with the non-living part of nature) is that these spontaneous or originative factors are in living organisms enabled in some way to assert themselves in a unified and effective manner" (Lillie, 1934, p. 314). It is this peculiar property of living organisms which is the basis both of the adaptive character of their complex inherited activities or instincts as exhibited under normal conditions of life and of the adaptive adjustments of these instincts that take place in the unusual situations of laboratory experiments.

SUMMARY

1. The external and internal anatomy of the larva of *Neuronia postica* are described briefly (Figs. 4–10).

2. The normal case as built in nature consists of rectangular segments of dead leaves or grass blades which are arranged in rings (Figs. 11 and 12).

3. The construction of new cases with normal material was studied in detail (Figs. 13–15). The larva always required some point of attachment to start constructing a new case. The cutting of a building unit was usually preceded by a preliminary 'sizing up' of the piece of material. During the whole building process the larva showed an efficient manipulation of the material by means of the pro- and mesothoracic legs.

4. When deprived of its case, the larva first constructed rapidly a rough preliminary sheath. Later on it added a more regular final case at the anterior end. The differences between the preliminary and the final cases appear to be caused mainly by the difference in the speed of construction.

5. The ability of the larvae to use strange building materials (the 'building range') was investigated (Figs. 16 and 17). The larvae built typical cases out of green leaves, onion skin, and cellophane; nearly typical cases out of paper and pine shavings. Very crude cases were built with tin foil, oatmeal, and barley. Twigs, pine needles, or pieces of wire were fastened loosely together but not used for the construction of a case.

Although the larvae were able to construct compact cases out of sand, they did not use this material readily. If pine needles and sand were given, the larvae used the former as a temporary scaffolding to which a sand structure was attached by a procedure that differed strikingly from the normal building process (Table I, Figs. 1–3).

6. In a series of experiments the larvae were given the choice between two building materials (Table II). During the early stages of construction the larvae always 'preferred' a material that could be used without cutting or was easily trimmed (small pine shavings, pieces of cellophane or paper, grass blades). In the construction of the final case, however, they showed a marked 'preference' for dead leaf, whether the latter was given whole or cut up into small pieces of approximate building-unit size (Expts. I–VII).

If given a mixture of small segments of leaf and paper, the larvae used both indiscriminately at first, the proportion of leaf and paper segments in the preliminary case depending on the relative abundance of the two materials; for the final case, comparatively more leaf segments were used (Expts. VII–IX, Fig. 18*A*–*C*).

The majority of the larvae 'preferred' cellophane to paper, even if the latter was supplied in ready-made building units and the former in a single large piece (Expts. X and XI, Table IV, Fig. 18*D* and *E*). This 'preference' seems to be due to the superior mechanical properties of the cellophane.

7. Following the amputation of either the first or the second pair of legs, the larvae were still able to construct fairly compact cases. They must, therefore, be capable of co-ordinating the movements of the remaining legs in such a way that orderly building activities result.

8. The question of whether or not the term 'instinct' as defined by Ziegler applies to the building activities of caddis-fly larvae is discussed briefly.

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PLATE I

Photomicrographs illustrating morphology of *Neuronia* larva. (See text, pp. 339–40.)

FIG. 4.—Larva removed from case, showing numerous filamentous gills and the small dragging hooks at posterior end of abdomen. ×1.8

FIG. 5.—Dissection of larva in physiological salt solution. Tracheae and the three portions of intestine are visible. ×1.8.

FIG. 6.—The same dissection after fixation in alcohol and acetic acid. Spinning glands visible as white, coiled tubes. The gonads are small white structures on either side of the mid-gut. Whole body cavity lined with leaflike fat-bodies. ×1.8.

FIG. 7.—Dissection of spinning glands. ×3.

FIG. 8.—Dissection of head showing ducts leading from spinning glands to lower lip near mid-ventral line, shape of mandibles and of pro- and meso-thoracic legs (the femora of both forelegs were perforated by dissecting pins). ×5.3.

FIG. 9.—Portion of spinning gland showing branching nuclei. Feulgen's stain, whole mount. ×53.

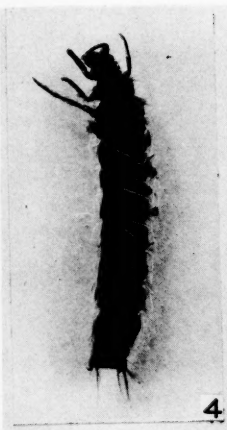
FIG. 10.—Different portion of gland from another larva. Secretion inside gland visible as faintly stained cylinder. ×53.

PLATE II

FIG. 11.—Different species of caddis-fly larvae collected in the same stream near Princeton. *A*, *B*, *E*, *F*, limnophilid larvae. *C*, *Neuronia postica; D*, *Phryganea* sp. (building units arranged in spiral). Slightly less than natural size.

FIG. 12.—Cases of young and old *Neuronia* larvae. Slightly less than natural size.

PLATE I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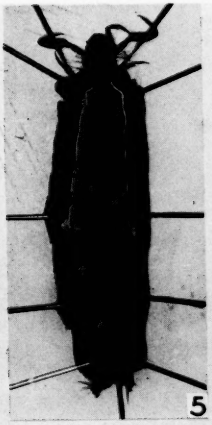
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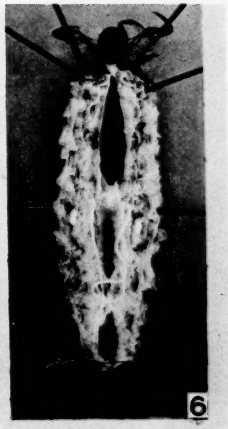
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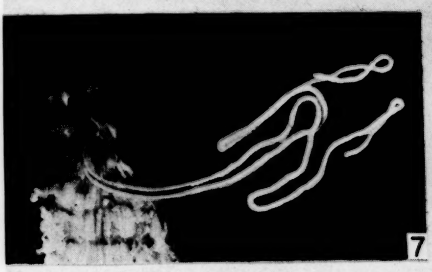
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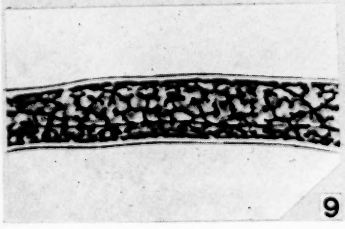
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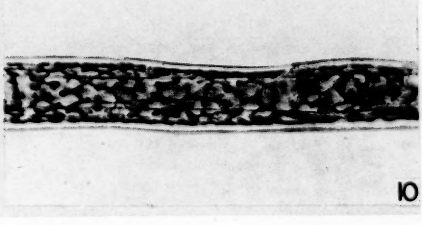
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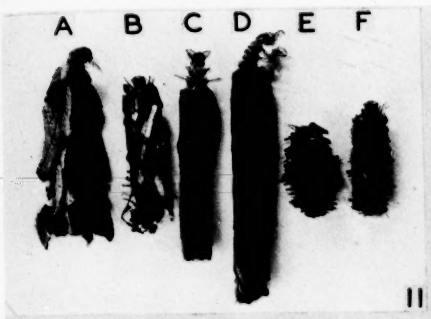
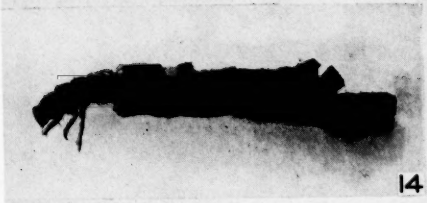
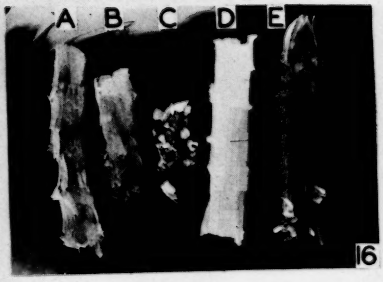
PLATE II







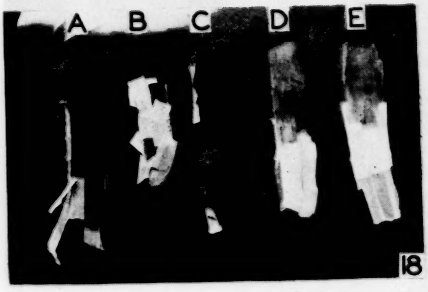


FIG. 13.—Original and new cases of a larva. New case (right) shows at posterior end part of the original pile of leaf fragments that served as a point of support. The building units are arranged more regularly at anterior end (beginning of final case). Slightly less than natural size.

FIG. 14.—Larva in rough preliminary case, $3\frac{1}{4}$ hours after removal from original case. ×1.6.

FIG. 15.—New case of another larva, 24 hours after removal from original case. At the anterior end (left) of the case the building units are arranged more regularly and form the first ring of the final case. ×1.4.

FIG. 16.—Building range of *Neuronia*. Cases built out of small pine shavings (*A*), cabbage leaf (*B*), sand (*C*), paper (*D*), and cellophane (*E*, the converging pieces at anterior end were added by larva to close case for pupation). Slightly less than natural size.

FIG. 17.—Portion of cellophane case photographed in transmitted light to show neat fitting of building units and irregular meshwork of dark silk threads lining the case. Posterior end of case to the right. ×4.7.

FIG. 18.—'Preference' experiments. Slightly less than natural size. *A*, dead leaf and paper given in small pieces and in equal amounts (table II, Expt. VII). Preponderance of leaf at anterior end of new case. *B*, dead leaf and paper given in small pieces in proportion 1:3 (table II, Expt. IX). Preliminary case, preponderance of paper. *C*, one piece of dead leaf 5 cm. square and paper in small pieces (Expt. V). Final case. Leaf is used almost exclusively. *D* and *E*, cellophane and paper given in small pieces and in equal amounts (Expt. X). The larvae preferred paper during the first hours but used cellophane exclusively later on.

FIG. 19.—Original case (left) and case built by larva after amputation of both prothoracic legs. See text (p. 351). Slightly less than natural size.

EVIDENCE FOR A SYMPATHETIC INNERVATION OF THE TELEOST HEART: WITH A NOTE ON A METHOD OF TRANSPLANTING THE HEART OF FUNDULUS EMBRYOS

(Eight figures)

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THE heart activity in all classes of animals—invertebrates and vertebrates—with the possible exception of the hagfishes, is regulated by the nervous system, although the beat originates in the cardiac muscle. In many of the invertebrates, especially the crustaceans (Conant and Clark, 1896) and arthropods (Carlson, 1906*a*), the heart is controlled by accelerator and inhibitory nerves. In the mollusks—*Mytilus*, *Mya*, *Venus*, *Hennites*, *Pecten* (Carlson, 1905)—octopus, and squid (Ransom, 1884) the heart is regulated by inhibitory fibers only; *Haliotis*, *Sycotypus*, *Natica*, *Lucapina*, *Aplysia*, *Bulla*, and *Pleurobrachia* contain only augmentor fibers, and in *Chiton* the heart is controlled by both sets of fibers (Carlson, 1905).

In the lower vertebrates, *Cyclostomata*, the question of the presence of regulator nerves is still unsettled. Green (1902) states definitely that in the California hagfish (*Polistotrema stoutii*) there are no cardio-regulator nerves for the systemic heart. Carlson (1904*b*) confirms Green's observations on the hagfish; but in a later paper (Carlson, 1906*a*) he found that the heart of the adult lamprey is controlled by augmentor and probably inhibitory fibers, although he failed to find these nerves in the larvae. This work may indicate that the previous work on the hagfish was done on larval forms.

In the elasmobranchs, Bottazzi (1901) showed that stimulation of the vagus in *Scyllium* and *Raja* produced a cardiac stoppage, but there was no effect on the heart rate by sympathetic stimulation. However, Izquierdo (1930) has shown that the heart of the same shark is under the control of the vagus and sympathetic nerves, as in other cold-blooded vertebrates.

There is an abundance of evidence in the teleost fishes that the vagus exhibits a powerful control over the heart. McWilliam (1884), Mills (1886), Bottazzi (1901), Armstrong (1931), and Brinley (1932) have shown that the hearts of various fishes can be arrested by either direct or reflex vagal stimulation. On the other hand, there is no positive evidence of a sympathetic innervation in the bony fishes. Von Brucke (1923) states that there is no evidence that the heart is controlled by accelerator fibers. In all animals above the fishes the heart is innervated by both sets of nerves.

A review of the subject on the nervous control of the heart in the various phyla of animals seems to indicate that no general rule can be laid down; but the presence of both sets of nerves in the elasmobranchs, amphibians, reptiles, birds, and mammals throws some doubt on the absence of cardio-accelerator fibers in the teleost fishes. Therefore, in connection with a study on the effect of drugs on the fish heart, it was thought advisable to study the action of certain substances, which are known to stimulate the sympathetic system in the higher animals, on the cardiac rhythm of embryonic and adult fish hearts in order to determine if the cardio-regulator nerves contain accelerator fibers. It is realized that no known drug is strictly specific in its action on the nerves of the cardiac apparatus, but nicotine and adrenalin in dilute concentrations approach this condition to a fair degree.

METHOD

In the following experiments eggs were stripped from female *Fundulus heteroclitus* and fertilized in the usual manner. The eggs were allowed to develop the required length of time, and the chorions were removed from the eggs the day before using. In the majority of the experiments the drugs were injected into the embryos free hand with a micropipette attached to a 1-cc. hypodermic needle. However, the same results were obtained by immersion of the naked embryos in the solutions, if the drugs were made up in isotonic salt solution or distilled water instead of sea water. This latter method was used in obtaining the nicotine curves. The embryos were immersed or injected at daily intervals from the time the heart started

to beat until hatching, and the rate of heart beat was determined by counting the number of beats per minute before and after treatment with the drugs.

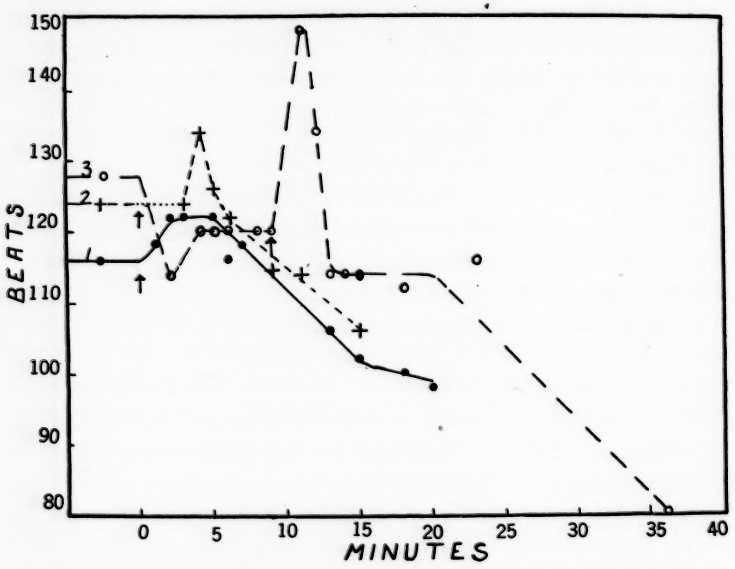

FIG. 1.—Graph showing the effect of nicotine, 1 part in 500 parts of isotonic NaCl solution, on the cardiac rhythm of *Fundulus* embryo. The arrows indicate the time of immersion. The number of beats per minute is plotted as the ordinate, and the abscissa indicates the time in minutes. (1) Embryos from 4 to 8 days after fertilization. (2) Embryos from 8 to 13 days after fertilization. The dotted line indicates the arrest following vagal stimulation. (3) Older embryos, immersed in atropine for 9 minutes previous to nicotine. The author wishes to thank Mr. Archie Solberg for making the slides from which the photographs were taken.

NICOTINE

Concentrated solutions of nicotine (pure alkaloid, Merk), 1 part to 500 parts of isotonic salt solution, produces a slight initial increase in the heart rate of the younger embryos (4–8 days after fertilization), which is immediately followed by a rapid decline terminating in diastolic stoppage (Fig. 1). In the case of the older embryos (8–13 days), the heart stops for a brief period within 1 or 2 minutes after treatment with the drug, this being due to vagal

stimulation (Brinley, 1932); the heart rate then shows a slight increase followed by a depression. The action of nicotine on the older heart, which has been freed from controlling action of the vagus by previous treatment with 0.5 per cent atropine solution, produces an initial acceleration in rate which surpasses the increase shown in the other experiments, and, as in the other cases, this acceleration is followed by a depression.

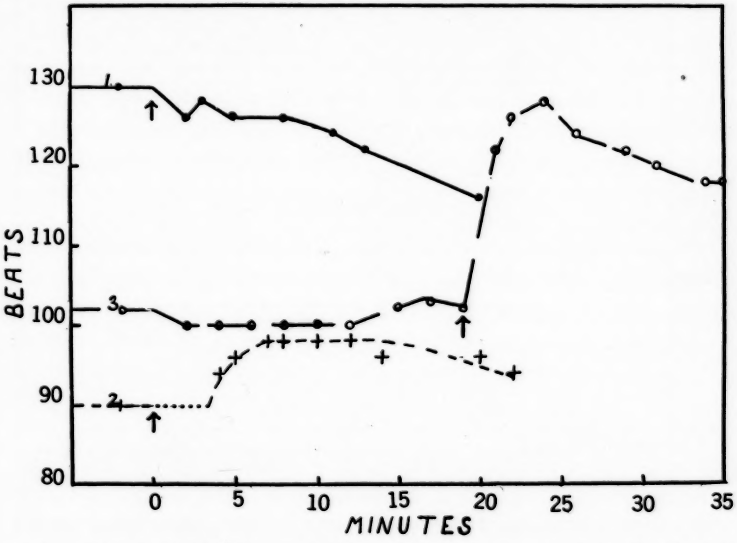

FIG. 2.—Graph showing the effect of nicotine, 1 part to 1,000, on the cardiac rhythm of *Fundulus* embryos. (1) Embryos from 4 to 8 days old. (2) Embryos from 8 to 13 days after fertilization. (3) Older embryos immersed in atropine for 19 minutes prior to nicotine.

The effect produced by a more dilute solution of nicotine, 1 part in 1,000, is different in some respects from the concentrated solution (Fig. 2). There is no stimulation and only a slight indication of a reduction in the rate of the younger hearts, indicating that nicotine at this concentration has little, if any, effect on the cardiac muscle. The hearts of the older embryos respond to the action of the drug by an initial arrest (vagal stimulation), followed by a gradual increase in rate, falling slowly to the previous value. The heart does not re-

spond to mechanical stimulation, which is evidence that the vagus has been paralyzed following stimulation. When the heart is freed from the vagus by atropine, the acceleration in rate is very pronounced, gradually returning to the normal rate.

The results obtained with very dilute solutions of nicotine, 1 part in 5,000 parts NaCl (Fig. 3), shows rather clearly that the augmentor

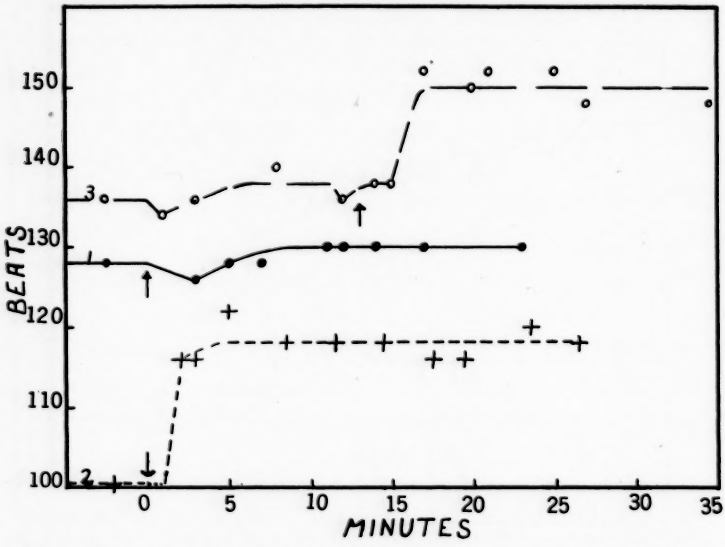

FIG. 3.—Graph showing effect of nicotine, 1 part to 5,000, on the cardiac rhythm of *Fundulus* embryos. (1) Embryos from 4–8 days after fertilization. (2) Embryos from 8–13 days. (3) Older embryos immersed in atropine for 13 minutes prior to nicotine.

fibers are stimulated by concentration of nicotine much lower than will stimulate the cardiac muscle or the inhibitor nerves. Nicotine in this dilution has no effect on the cardiac muscle prior to innervation and does not inhibit the heart by vagal stimulation; as the heart still responds to mechanical stimulation after it has been immersed in the drug.

It may be seen from a study of the data presented that the behavior of the embryonic hearts of *Fundulus* toward nicotine depends upon the age of the embryo and the concentration of the drug. The reaction of the young embryos from 4 to 8 days old is decidedly

different from the behavior of the embryos from 8 days old until hatching age (12 days). This difference is taken to indicate that the sympathetic nerves innervate the heart on the eighth to tenth day. Therefore, the hearts of the embryos just before hatching contain the double innervation. Using this assumption as a working hypothesis, the following conclusions become apparent. Concentrated solutions produce an initial stimulation of the cardiac muscle, followed by a depression and a final arrest in the diastolic condition. There is also a decided initial stimulation of the vagus in the older embryos which rapidly passes into paralysis. The stimulation of the sympathetic system, as indicated by acceleration in the heart rate, is largely suppressed by the inhibitory action of the vagus but becomes apparent when the controlling influence of the vagus is removed by atropine. There is no increase in heart rate after immersion of the embryos in atropine; therefore, the increase in heart rate after immersion in nicotine cannot be accounted for by removing the controlling influence of the vagus. A more dilute solution (1 part to 1,000) of nicotine does not produce stimulation of the cardiac muscle. However, there is a marked stimulation and paralysis of both sets of cardiac nerves. Dilute solutions of nicotine (1 part to 5,000) do not stimulate the cardiac muscle; and there is no evidence of a vagal stimulation or paralysis as the heart responds to mechanical stimulation while the embryo is immersed in the drug, but there is a characteristic acceleration in the heart rate, which is evidence for sympathetic stimulation. The increase in rate is maintained for a long period of time, indicating that paralysis does not follow stimulation. In all experiments using nicotine in dilutions as low as 1 part in 100,000 parts of isotonic salt solution the vagus becomes decidedly more sensitive to mechanical reflex stimulation. The vibration of the room or the placing of the watch glass on the stage of the microscope is sufficient to temporarily arrest the heart unless great care is taken. The increase in susceptibility is very important, as it may be erroneously taken for stimulation of the vagus.

ADRENALIN CHLORIDE

A minute droplet of adrenalin chloride, from 1 part in 1,000 to 1 in 50,000 of distilled water, was injected into *Fundulus* embryos (free from corion) anteriorly to the vitelline arteries. The immedi-

ate contraction of the melanophores on the dorsal surface of the embryo is a positive criterion of a successful injection. Injections were made into embryos at daily intervals from 4 days until hatching. Controls were conducted by injecting distilled water into the embryos, and in no case did any change occur in the rate of contraction except when large amounts were given. The rate of cardiac contractions was determined by counting the number of beats per minute before and after injection of the drug.

Young embryos.—Injections into embryos from 4 to 7 days old, inclusive, do not produce any change in the rate or rhythm of the heart. This indicates that adrenalin has no visible effect on the rate of contraction of the embryonic cardiac muscle.

Older embryos.—Figure 4 shows the results obtained by injecting adrenalin into embryos 8–13 days old, inclusive. The heart of the older embryos stops for several seconds immediately after injection, owing to a reflex stimulation of the vagus. Following this arrest and in all other cases, the heart rate quickly increases 10–20 per cent above the rate prior to injection and gradually returns to its initial value. This acceleration is always obtained if the normal rate of the heart is less than 140 beats per minute, but no alteration in rate occurs if the heart is beating at a faster rate. Therefore, there seems to be a maximum rate to which the heart can be stimulated. This marked acceleration seems to indicate either that the heart is innervated by augmentor nerves on the eighth day after fertilization or that the cardiac muscle of the older embryo is decidedly different physiologically from the younger embryos, as no effect of the drug is shown on the cardiac muscle prior to the eighth day of incubation.

Antagonistic action of adrenalin and ergotoxin.—To obtain more data on the effect of adrenalin on the heart mechanism, a series of experiments were conducted by injecting adrenalin into embryos which had previously been injected with ergotoxin and also treating embryos with ergotoxin after adrenalin. Ergotoxin is a well-known sympathetic depressant and is used in physiological experiments to free the organism from the controlling influence of the sympathetic system. In the present experiments ergotoxin in concentrations varying from 1 part in 1,000 to 1 part in 10,000 parts of distilled water was injected in *Fundulus* embryos from 8 to 13 days after

fertilization. This injection was followed within 5–10 minutes with an injection of adrenalin. In no case was there any acceleration in the heart after adrenalin injection followed ergotoxin. If the administration of ergotoxin followed adrenalin, there was an immediate termination of the acceleration and the rate of cardiac rhythm re-

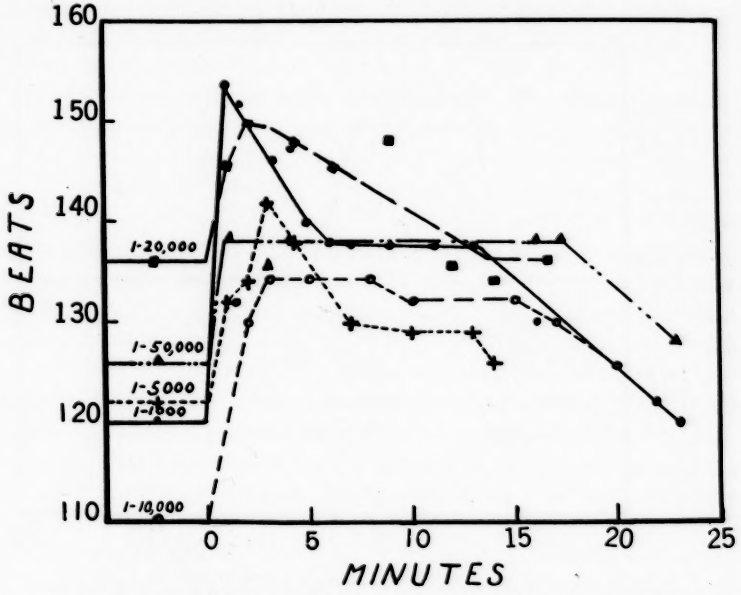

FIG. 4.—Graph showing the acceleration in the rate of heart beat following the injection of adrenalin chloride into *Fundulus* embryos from 8 to 13 days after fertilization. Injections made at the time indicated by the arrow. The number of beats per minute is plotted as the ordinate, and the abscissa indicates the time in minutes after injection.

turned to normal. (Fig. 5). Therefore, it seems apparent that the acceleration in rate of the heart beat after injection of adrenalin is due to stimulation of the augmentor fibers and not to a direct action on the cardiac muscle.

A METHOD OF TRANSPLANTING THE HEART OF *Fundulus* EMBRYOS

In connection with the foregoing investigation it is realized that the young embryonic heart is a generalized tissue and that specializa-

tion and differentiation occur as the heart develops. Therefore, it is desirable to know if there are any effects of the drugs on the cardiac muscle as specialization and development occurs, and if there is a time during differentiation when the cardiac muscle would become sensitive to the action of the drugs. It was thought that a certain amount of information along this line may be obtained if the heart

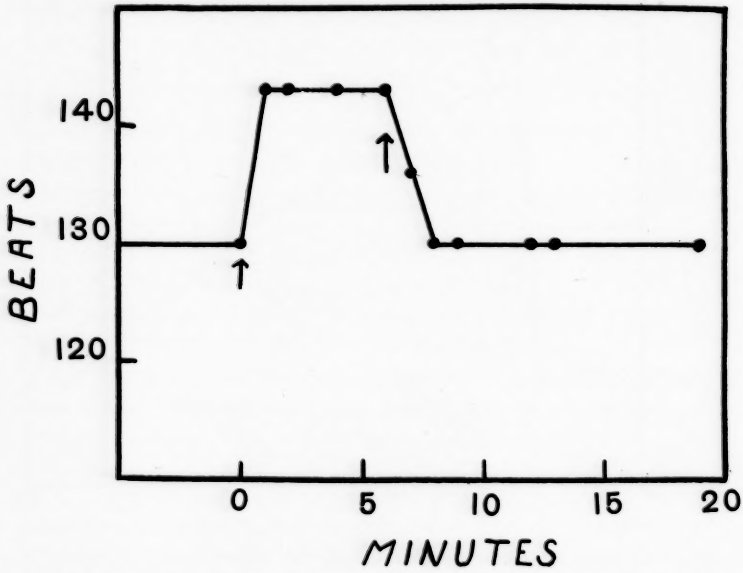

FIG. 5.—Graph showing the effect of injection of adrenalin, 1 part to 1,000, time indicated by first arrow; followed by injection of ergotoxin, time indicated by second arrow.

muscle could be cultured free of the cardiac nerves. After many negative trials with balanced salt solutions as a culture medium, it was found that the most satisfactory method of cultivating the excised heart was to implant the pulsating organ into a host embryo in such a way that it could be easily observed and would not be readily innervated. The implanted heart grew rapidly and contractions continued for several days; but unfortunately there seemed to be considerable dedifferentiation and no specialization occurred, so that very few data are obtained on the foregoing problem; but as this method has opened up several problems in relation to the be-

havior of the embryonic heart it appears desirable to present the technique to those interested.

In this investigation the two species of marine *Fundulus* were used: *F. heteroclitus* as the donor and *F. majalis* as the recipient. *Fundulus heteroclitus* was used as the donor because it was the species used in the previous experiments. The larger size and the slowness of development of the eggs of *F. majalis* made this species a very satisfactory host.

Preparation of the heart for transplanting.—The eggs of *Fundulus heteroclitus* were stripped from the females, fertilized in finger bowls, and allowed to develop. Chorions were removed from eggs from 5 to 12 days after fertilization. The naked embryos were then placed in 60 per cent sea water (60 cc. sea water plus 40 cc. distilled water), which is isotonic with the excised heart. The vitelline membrane is then cut with a fine-pointed scalpel (dissecting needle) in front of the heart where the membrane unites with the embryo. The vitelline membrane can then be carefully pulled away from the anterior portion of the yolk sac. This operation leaves the heart exposed but still attached to the blood vessels and beating normally. The aorta is then cut where it joins the embryo, and by gently elevating and pulling the tip of the ventricle the heart can be freed from the yolk sac and the auricle can then be severed from the vein. If this is carefully done, the yolk sac will not be broken and the heart will be free of all foreign tissue and will not be imbedded in the gelatinous yolk which seems to prevent normal contraction. The excised heart will continue to beat for 2 hours in 60 per cent sea water. The entire heart can be successfully transplanted at any time during this interval, but in these experiments the heart was cut at the auricular-ventricular junction and the auricle transplanted as soon as possible after removal from the donor. The ventricle stopped beating very soon after separation from the auricle.

Preparation of the recipient.—The chorions were removed from the eggs of the recipient at least a day before the implant was made, and only the embryos in good condition at the end of that time were used. The naked embryos were placed in 60 per cent sea water, and a very small slit was made through the vitelline membrane adjacent to the embryo, laterally of the mid-gut and just posterior to the vitelline artery. The pulsating auricle previously prepared was

immediately transferred by teasing needles to the slit made in the vitelline membrane of the host, and held in a horizontal position (Fig. 6) for a few seconds until the membrane formed around the implant and securely held it in place. Precautions must be taken not to push the implant into the yolk so that it will be submerged when the vitelline membrane is repaired.

Growth and behavior of the implant.—In the majority of cases the heart did not stop beating while being removed from the donor and transferred to the host. In some cases, however, the implanted heart stopped within 2 or 3 hours after transplanting, but contractions

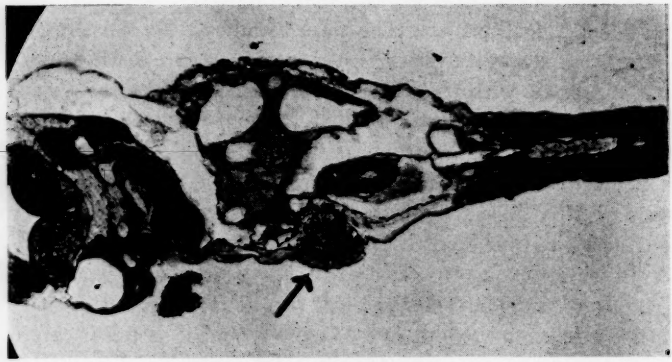

FIG. 6.—Low-power photograph of a frontal section of a *Fundulus* embryo, the arrow showing the location of implanted heart. Twenty hours after transplanting.

were again resumed after the tenth or twelfth hour. For the first 24 hours the cardiac rhythm was slow but regular at 25–30 beats per minute. The rate gradually increased until on the third day the rate approached the normal host heart, 135–50 beats per minute. There was considerable growth of new tissue during the first day. Large globular dedifferentiated cells appeared on the surface of the pulsating implant, and many of these cells sloughed off and could be picked up with teasing needles. These isolated cells were studied in the living condition under the low and high powers of the microscope, but no contraction could be seen. These isolated cells are very interesting, and further study is in progress.

Sectioned and stained materials (Delafield's haematoxylin) showed that the center of the mass of tissue is composed of rather loosely packed cells of large size with prominent nuclei (Fig. 7). To

all appearances these were identical with the cells of the host heart. Many large flask cells could be seen adhering to the periphery of the mass (Fig. 8). These were the cells that appeared on the surface in the living condition. A few cells were undergoing mitosis. It is very difficult to identify this new growth of embryonic tissue, but it is believed to be largely myoblasts.

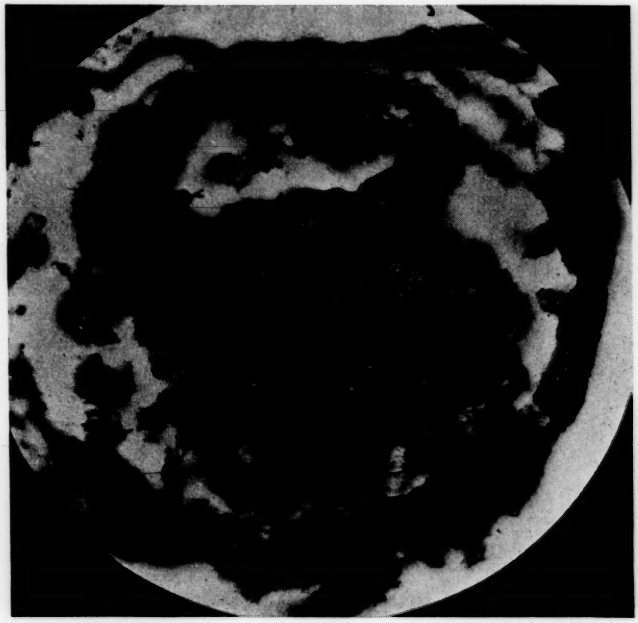

FIG. 7.—Photomicrograph (2 mm. lens) of the implanted heart, showing the original lumen of the auricle and new growth of tissue. Four hours after transplanting.

In many cases the original lumen of the heart retained its identity after transplantation and original erythrocytes could be seen in the stained sections. In one case a blood capillary grew into the pulsating tissue.

Action of adrenalin on the transplanted heart.—In order to throw more light upon the question of the differential effect of adrenalin on the cardiac nerves and muscle, a series of experiments were conducted on the action of the drug on the rhythm of the embryonic heart after severing the nerves and transplanting the heart into

another embryo in such a manner that the heart would continue to beat but would not likely be innervated. After the transplanted heart had established a uniform rhythm of about 140 beats per minute, the host was injected with adrenalin and the rate of contraction of the transplanted heart was determined. In about 90 per cent of the cases the transplanted heart showed no effect of the drug; but

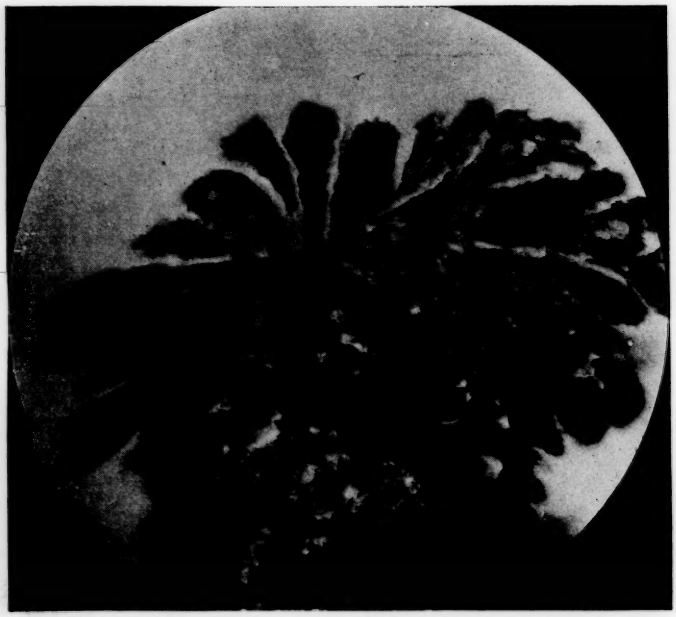

FIG. 8.—Photomicrograph (2-mm. lens) of the periphery of an implanted heart, showing new growth of myoblasts. Four hours after transplanting.

in the remaining 10 per cent the heart rate was greatly accelerated, duplicating the results obtained with the innervated heart. No explanation of the acceleration is offered, unless that in an occasional heart the nerve-endings of the sympathetic system may be carried over and remain functional in the transplanted heart.

SUMMARY

A study was made on the effect of nicotine and adrenalin solutions on the cardiac rhythm of *Fundulus* embryos. The embryos varied in age from 4 to 13 days. The acceleration in rate of the heart beat of

the older embryos (8–13 days) immediately following the injection or immersion in dilute nicotine solution or adrenalin is considered positive evidence for a sympathetic innervation of the teleost heart, as no acceleration occurs in embryos younger than 8 days or in older embryos which were freed from the controlling action of the sympathetic nerves by previous treatment with ergotoxin. Adrenalin also shows no effect on transplanted hearts. Therefore, it seems very likely that the sympathetic system innervates the teleost heart on or about the eighth day after fertilization at room temperature of 22°–24° C.

A method is described of transplanting the embryonic heart of *Fundulus heteroclitus* to *F. majalis*. The implant continued beating for several days; considerable growth and dedifferentiation occurred.

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HEAD FREQUENCY IN EUPLANARIA MACULATA IN RELATION TO THE NERVOUS SYSTEM[1]

(Twenty-three figures)

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IN AN earlier paper (Child and Watanabe, 1935) evidence was given showing that the relation between head frequency in the reconstitution of isolated pieces of *Euplanaria dorotocephala* and length of piece, level of body, and time of anterior and posterior section results from a physiological factor originating in connection with the posterior cut surface. This factor inhibits head development differentially to a greater or less degree and determines the teratophthalmic, teratomorphic, anophthalmic, and acephalic forms. On the basis of the evidence presented, it was suggested that the factor inhibiting head development is probably nervous in character and results primarily from section of the nerve cords at the posterior end of the piece.

The present paper is a further analysis of head frequency and is particularly concerned with the question of the relation of head frequency to a nervous factor. The writer is much indebted to Professor C. M. Child for his suggestions and criticisms.

MATERIALS AND METHOD

The species identified by Hyman (1931) as *Euplanaria maculata* served as material. Unpublished data obtained in this laboratory have shown that in this species, as in *E. dorotocephala*, head frequency varies with length of piece, level of body, and relative time of anterior and posterior section. For the operations undertaken in experiments described below, this species is somewhat more favorable material than *E. dorotocephala* because it is less active and because the death frequency in short pieces is lower.

Some ten years ago Šivickis (1923) presented data on head fre-

[1] Support for this work was provided from a grant by the Rockefeller Foundation to the University of Chicago in aid of research in the biological sciences.

quency in relation to length of piece and level of body in a species which he named *Planaria* (*Euplanaria*) *lata*. Recently Hyman (1931) has maintained that this form is *Euplanaria maculata*. If this is the case, it would be unnecessary to give further data concerning those points here; but according to Šivickis' figures and description, *E. lata* differs considerably from *E. maculata* in external appearance, shape of body, position of the zone of fission, and morphology of the genital system. Moreover, Dr. Child has informed me that in a mixed stock of *E. lata* and *E. maculata* from the same locality, the two forms could easily be distinguished by the naked eye. During recent years conditions have been much changed by the frequent removal of aquatic vegetation from the park lagoon from which Šivickis' material was obtained, and it has not been possible to find the form which he described as *Planaria lata;* consequently, a further check on this point is at present impossible. In view of this situation it seems necessary to give data on head frequency in relation to length of piece and body-level in the species identified as *E. maculata*.

For the experiments, animals 12–16 mm. long (the average length of full-grown animals) which were without visible sex organs and which had not recently undergone fission were selected and were kept without food 7–12 days before operation. For experiments on the effect of delay of section (Ser. II), pieces 1.5 mm. in length from a level immediately posterior to the mouth were used because, under the usual conditions, pieces from this body-level show a lower head frequency than pieces of the same length from other levels, and the experimental conditions increased head frequency. In other experiments concerned with the effect of the nervous system in inhibiting head development, the same region of body and the same distance (1.5 mm.) between anterior and posterior section were used.

An M/100 solution of chloretone was found most satisfactory as anesthetic. Ten minutes' exposure to this solution induced complete anesthesia, and general motor and sensory recovery occurred in intact animals in 10–15 minutes after return to water. The death-rate with this treatment was less than 5 per cent. Exposure to this concentration for 25 minutes or more results in a high death-rate. Other concentrations, M/50 and M/200, were tried; but in the former more or less disintegration usually occurred after return to water

and in the latter complete loss of motility occurred very slowly, sometimes only after 2–3 hours or more. Such long exposures were not suited to the experiments undertaken, since it was desired to observe separately, as far as possible, the effects of the anesthetic and of delay of section. The details of operation and treatment by chloretone differ in different experiments and will be given below for each case.

Each experimental lot consisted of 20 pieces in 200 cc. of water kept in finger bowls in darkness without change of water. Fourteen days after operation the numbers of normal, teratophthalmic, teratomorphic, anophthalmic, and acephalic forms (Child, 1921) were recorded. A numerical value for the head frequency of each lot was obtained by the method used in another paper (Child and Watanabe, 1935) and expressed by the formula

$$\frac{100n_5+80n_4+60n_3+40n_2+20n_1+n_0}{N}.$$

In this formula n_5– n_1 are the numbers of pieces which develop, respectively, as normal, teratophthalmic, teratomorphic, anopthalmic, and acephalic forms; and n_0, the number of dead pieces. The numbers 100, 80, 60, 40, 20, and 0 are numerical values or coefficients arbitrarily assigned to the different head-forms. N is the total number of pieces in the lot or series of lots. It may be pointed out that dead pieces (n_0) are included in this formula because under certain experimental conditions death frequency, like head frequency, shows definite distribution in relation to length of piece and body-level. When no such definite distribution occurs, n_0 may be omitted if the death-rate is very low; but conditions which give a high death-rate are obviously not entirely satisfactory for determination of head frequency. In the experiments of this paper, deaths occurred only in Series V after chloretone, and in that series were less than 5 per cent.

EXPERIMENTAL DATA

Series I. Head frequency in relation to length of piece and body-level.—In these experiments the head frequency of 1/4, 1/6, and 1/8 pieces of the postcephalic region was determined. As in the earlier

paper (Child and Watanabe, 1935), the pieces are designated according to the fraction of the postcephalic body length and the body-level which they represent, 1/4–1—1/4–4, 1/6–1—1/6–6, and 1/8–1—1/8–8, the order being from anterior to posterior levels. Room temperature ranged from 19° to 21° C., during the experiments. Head frequencies were determined in five lots of 20 pieces each—a total of 100 pieces for each length and each body-level. Maximum, minimum, and average frequencies for each group of five lots calculated according to the formula given above appear in Table I. The maximum and minimum frequencies are given in order to show the

TABLE I

HEAD FREQUENCY IN 1/4, 1/6, AND 1/8 PIECES OF *Euplanaria maculata*

Each average frequency given is the average of five lots of 20 pieces each—100 pieces.

Pieces	Maximum	Minimum	Average	Pieces	Maximum	Minimum	Average
1/4–1	100	100	100.0	1/8–1	100	98	98.8
1/4–2	87	75	81.2	1/8–2	87	70	82.0
1/4–3	100	90	97.6	1/8–3	76	50	64.2
1/4–4	100	100	100.0	1/8–4	66	44	52.8
				1/8–5	81	67	71.6
1/6–1	100	99	99.8	1/8–6	97	85	92.0
1/6–2	91	77	81.5	1/8–7	100	95	98.4
1/6–3	67	54	61.2	1/8–8	100	100	100.0
1/6–4	97	79	85.0				
1/6–5	100	97	98.4				
1/6–6	100	100	100.0				

range of variation in each group. Figure 1, graphed from the average frequencies, shows that head frequency decreases from the anterior level to the middle region and increases again at more posterior levels, and that the shorter the pieces the greater the decrease and increase in frequency. The graph resembles closely the head-frequency graphs of *Euplanaria dorotocephala* (Child and Watanabe, 1935), but the frequencies in the middle region of the body are in general somewhat higher in *E. maculata*.

It may be noted that a few bipolar forms appeared in the 1/8 pieces: 2 from 1/8–5, 2 from 1/8–6, and 5 from 1/8–7.

Series II. Effect of delay of anterior or posterior section on head frequency.—It was shown by Child and Watanabe (1935) for *Euplanaria dorotocephala* that at body-levels which show a low head

frequency in short pieces the frequency increases progressively with increasing delay of anterior or posterior section. Similar experiments show the same effect in *E. maculata*.

For the experiments on delay of posterior section, the anterior section was made at the level just posterior to the mouth and the posterior section 1.5 mm. posterior to it, in one set of pieces immediately, in another 2 hours, in a third 4 hours, and in other sets every 2

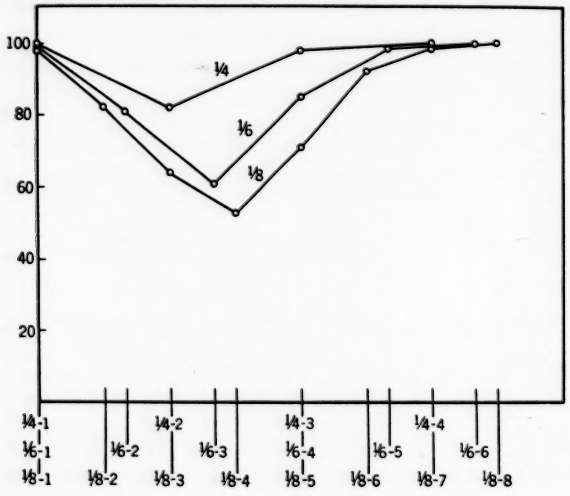


FIG. 1.—Head frequency in 1/4, 1/6, and 1/8 pieces of *Euplanaria maculata*. Ordinates are mean head frequency values calculated by formula given in text; abscissae are body-levels.

hours up to 14 hours after the anterior section. Five similar experiments were performed with 20 pieces each for each period of delay—a total of 100 pieces for each period except for 12 and 14 hours' delay of posterior section, where the totals are 40 each. Room temperatures were 19°–22° C. The average frequencies for each period are graphed in Figure 2, which shows a continuous and rapid increase in head frequency up to 6 hours' delay and 100 per cent normal heads with 10 hours' delay of posterior section.

In determining the effect of delay of anterior section, the same body-level, length of piece, and the same number of pieces for each period of delay were used; but, since it was known that in *E. doro-*

tocephala increase in head frequency occurs much more slowly with delay of anterior section than with delay of posterior section, the periods of delay were made much longer: 12, 24, 36, 48, and 60 hours. Room temperatures were 22°–25° C. The average frequencies are graphed in Figure 3.

Comparison of Figures 2 and 3 with graphs for *E. dorotocephala* (Child and Watanabe, 1935, Figs. 7, 8) shows a close similarity for the two species. With delay of posterior section the frequency reaches 100 per cent earlier in *E. maculata*, but this difference may depend in part on the length of piece chosen for the experiment and

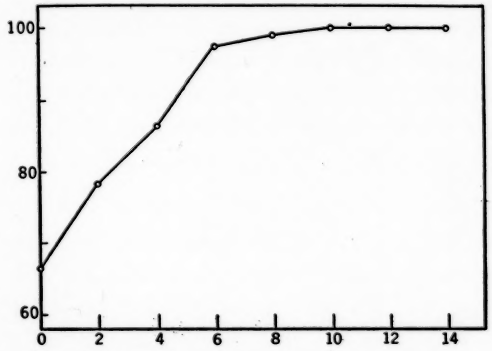


FIG. 2.—Increase of head frequency with delay of posterior section in pieces 1.5 mm. long from the region posterior to the mouth. Ordinates are head-frequency values; abscissae, delay of posterior section in hours.

in part on the fact that the head frequency for a given fraction of body length is somewhat higher at the level immediately posterior to the mouth in *E. maculata* than in *E. dorotocephala*. With delay of anterior section, both species show first a slight decrease and then an increase in head frequency. Discussion is postponed until other data have been presented.

In these experiments also a few bipolar forms appeared, but the number was too small and the distribution too irregular to permit any conclusions concerning possible relation to the period of delay of section. In the experiments on delay of posterior section, 52 bipolar forms were observed; in those on delay of anterior section, 17.

Series III. Effect on head frequency of anterior or posterior section under anesthesia.—Using pieces of the same length and from the

same level as in the preceding series, the effect on head frequency of making the anterior or posterior section under chloretone anesthesia was determined. In the experimental lots intact animals were placed in M/100 chloretone for 10 minutes, with complete loss of motility. Then the posterior or anterior section was made in the chloretone solution, and the pieces remained in the solution for another 10 minutes in order to make more certain the decrease or elimination of nervous stimulation immediately following section. Next, pieces were washed three times and left in water. After 20 minutes in water, the second section, anterior or posterior, was made.

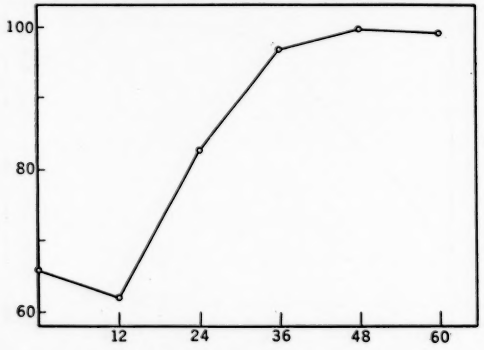


FIG. 3.—Increase of head frequency with delay of anterior section in pieces 1.5 mm. long from the region just posterior to the mouth. Ordinates are head-frequency values; abscissae, delay of anterior section in hours.

Two control lots were made for each experimental lot. In the first, the second section (anterior or posterior) was made 30 minutes after the first (posterior or anterior), without the use of chloretone (Control I). In the second (Control II), intact animals were kept in chloretone for 20 minutes, then returned to water, and 20 minutes later (that is, after apparently complete recovery) the first section (posterior or anterior) was made, and 30 minutes after the first section the second section (anterior or posterior) was made.

The head frequencies are given in Table II. Considerable variation appears in different control and experimental lots, but in all cases the experimental lots show a higher frequency than the corresponding controls. The averages of Controls I and II differ but

little. In the cases of posterior section under anesthesia, the experimental average is 26.8 higher than that of Control I and 25.4 higher than that of Control II. In those of anterior section under anesthesia the experimental average is 25.3 higher than that of Control I and 23.5 higher than that of Control II. In other words, the increase in

TABLE II

HEAD FREQUENCIES AFTER FIRST (ANTERIOR OR POSTERIOR) SECTION UNDER ANESTHESIA AND DELAYED (POSTERIOR OR ANTERIOR) SECTION AFTER RECOVERY

Control and experimental lots consist of 20 pieces each.

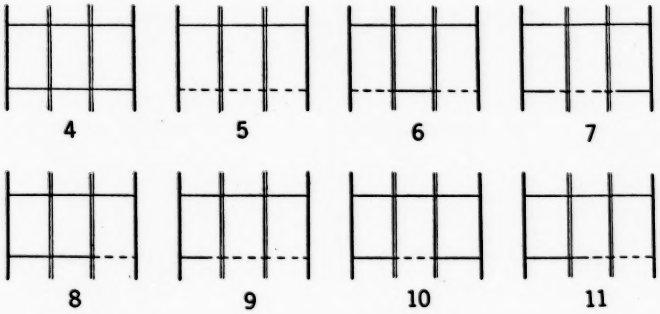
Lot	Control I	Control II	Experimental
	Posterior Section under Anesthesia		
1	38	43	60
2	52	61	84
3	54	63	89
4	64	61	81
5	71	58	96
Avs.	55.8	57.2	82.6
	Anterior Section under Anesthesia		
1	50	67	93
2	56	60	76
3	63	57	75
4	66	58	92
Avs.	58.7	60.5	84.0

head frequency is about the same in both series of experiments. The experiments of Series II show that 30 minutes' delay of either anterior or posterior section increases head frequency very little or, in case of delay of anterior section (Fig. 3), not at all. The data of Table II show that the brief period of anesthesia, together with 30 minutes' delay of either anterior or posterior section, does increase head frequency and is still effective 30 minutes after return to water. Even though recovery is apparently complete within that time,

posterior section is less effective in inhibiting head development than without preceding anesthesia. These results support the suggestion made in an earlier paper (Child and Watanabe, 1935) that the factor inhibiting head development in short pieces is primarily nervous in character. If this is the case, it seems probable that it results, in part, perhaps chiefly from section of the nerve cords at the posterior end of the piece, though section of other parts of the nervous system may also contribute to the effect. It is highly probable that the effect of the anesthetic is greater and recovery slower in the region of the cut surface where the full concentration is in direct contact with the nervous tissues. In the experiments of Series III this region is either posterior or anterior, but in either case is still effective in blocking the factor inhibiting head development after 30 minutes in water with general motor and sensory recovery.

Series IV. Effect on head frequency of delay of section of the nerve cords at the posterior end of the piece.—It was shown above (Ser. II, Fig. 2) that delay of posterior section increases head frequency in short pieces and that with 6 hours' head frequency is increased almost to 100 per cent (Fig. 2). In the present series the attempt is made with pieces of the same length and from the same body-level as Series II to determine the effect of 6 hours' delay of section of the nerve cords. Figures 4–11 show the various operations. In these figures the transverse unbroken lines indicate the cuts made first; the broken lines, the cuts made 6 hours later. The two vertical double lines in each figure indicate the nerve cords. For comparison with the experimental pieces two controls were made: one (Control I) with anterior and posterior section made at the same time (Fig. 4), the second (Control II) with posterior section made 6 hours later than the anterior (Fig. 5). In experiment *A* (Fig. 6) section of the two nerve cords and the region between them was made at the same time as the anterior section; in Experiment *B* (Fig. 7) section of the regions lateral to the nerve cords was made at the same time as the anterior section, and the nerve cords were cut 6 hours later. In Experiment *C* (Fig. 8) section of both nerve cords and one lateral region was made at the same time as anterior section; and in Experiment *D* (Fig. 9), 6 hours later. In Experiment *E* (Fig. 10) section of both nerve cords and both lateral regions was made at the same time

as anterior section, and section of the median region between the nerve cords was made 6 hours later. In Experiment *F* (Fig. 11) section of one nerve cord and one lateral region, that is, a half-transverse section, was made at the time of anterior section; and the other half-section, 6 hours later. The purpose of Experiments *E* and *F* is comparison of the effect of section of both nerve cords and both lateral regions at the time of anterior section with the effect of 6 hours' delay of section of one nerve cord and one lateral region.



FIGS. 4–11.—Different operations in experiments on effect on head frequency of delay of posterior section of nerve cords and other parts. Transverse unbroken lines represent the first cuts; broken lines, cuts made 6 hours later. Double vertical lines represent nerve cords. Figures 4 and 5 are controls; Figures 6–11, Experiments *A*, *B*, *C*, *D*, *E*, *F* (Table III).

Head-frequency values are given in Table III. In Control I with anterior and posterior section made at the same time, the head frequency is 66; in Control II with posterior section delayed 6 hours, it is 98. These results are similar to those given in Figure 2. In the experimental lots head frequency is increased in every case by 6 hours' delay of section of both nerve cords (*B*, *D*) or of one (*F*); and in *B* and *D* is as high as in Control II, in which the whole posterior section is delayed 6 hours. The average increase in *B* over *A* is 19; in *D* over *C*, 17. In Experiment *F*, in which section of one nerve cord is delayed, the increase over experiment *E*, in which both nerve cords are cut at the time of anterior section, is less, only 9.8. The data show consistently that delay of section of both nerve cords or of one increases head frequency. The results of Experiment *F*

agree with the results of posterior half-section in *Euplanaria dorotocephala* (Child and Watanabe, 1935, pp. 21–26) in indicating that when only one nerve cord is sectioned posteriorly the cells on the side of the anterior cut surface with intact nerve cord compensate the inhibition of development in the cells on the side with sectioned nerve cord.

The data of Table III bring to light another point of importance. In Experiments *A*, *C*, and *E*, in which both nerve cords are cut at the time of anterior section but both lateral regions (Expt. *A*), one lateral region (Expt. *C*), or the median region (Expt. *E*) remain un-

TABLE III

EFFECT ON HEAD FREQUENCY OF 6 HOURS' DELAY OF SECTION OF NERVE CORDS AT THE POSTERIOR END OF THE PIECE

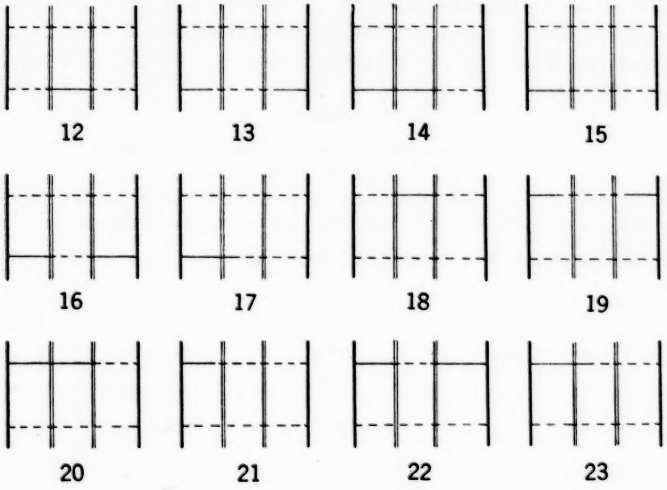
Each lot consists of 160 pieces for each control and experiment.

Lot	Controls		Experiments					
	I	II	A	B	C	D	E	F
1	62	99	80	98	80	99	83	93
2	64	97	85	98	75	97	77	92
3	64	100	78	93	86	97	84	95
4	67	99	70	95	78	96	88	94
5	73	95	76	100	85	100	84	91
Avs.	66.0	98.0	77.8	96.8	80.8	97.8	83.2	93.0

cut for 6 hours, head frequencies are higher than in Control I in every lot. This difference suggests that complete posterior section (Control I) inhibits head development to a greater degree than the various types of partial section (*A*, *C*, *E*), even though they involve section of the nerve cords. Apparently, section of other regions as well as of the nerve cords plays some part in inhibiting head development, though the data indicate that section of the nerve cords is the more important factor. Parts of the nervous system are, of course, present in regions lateral and median to the nerve cord; and it seems possible in the light of the data at hand that section of these parts has some effect in inhibiting head development. It is also possible, however, that when the nerve cords are cut, contact, and perhaps

some degree of union of tissues across the section during the 6 hours before section is completed, decreases the effect of section on the nervous system and so increases head frequency.

In this series, as in other similar series of short pieces, a few bipolar forms occurred: 8 in Control I, 16 in Control II, 9 in Experiment *A*, 12 in *B*, 7 in *C*, 10 in *D*, 3 in *E*, and 5 in *F*.



FIGS. 12–23.—Operations in experiments on effect on head frequency of posterior and anterior section of nerve cords and other parts under anesthesia and in the anesthetic and completion of isolation after 20 minutes in water following anesthesia. Unbroken transverse lines represent cuts made under anesthesia and in the anesthetic; broken lines, the cuts made in water after anesthesia. Figures 12–17, Experiments *C–H*, *J*, *K*, *L*, *M* (Table IV); part of posterior section in anesthetic. Figures 18–23, Experiments *N*, *P*, *Q*, *R*, *S*, *T* (Table V); part of anterior section in anesthetic.

Series V. Effect on head frequency of posterior and anterior section of the nerve cords under anesthesia.—In this series of experiments intact animals were kept in M/100 chloretone for 10 minutes; partial posterior or anterior section was then made in the chloretone solution; the animals remained in chloretone for 10 minutes after partial section, were then returned to water; and, 20 minutes later after apparent recovery, isolation of pieces 1.5 mm. in length was completed. Figures 12–17 show the experiments on partial posterior

section under anesthesia; and Figures 18–23, the experiments on partial anterior section under anesthesia. In each of these figures unbroken transverse lines indicate the parts of the posterior or anterior cut made under anesthesia and broken lines the parts made 30 minutes later, after 20 minutes in water. Vertical double lines represent the nerve cords.

The head frequencies after partial posterior section under anesthesia and completion of posterior section and entire anterior section 30 minutes later, after 20 minutes in water, are given in Table IV.

TABLE IV

EFFECT ON HEAD FREQUENCY OF PARTIAL POSTERIOR SECTION UNDER ANESTHESIA

Each lot consists of 40 pieces, 20 pieces for each of two experiments of the lot.

Lot	Experiments		Lot	Experiments		Lot	Experiments	
	G	H		J	K		L	M
1.....	71	50	6.....	66	46	11.....	55	48
2.....	81	48	7.....	75	56	12.....	70	31
3.....	84	39	8.....	78	60	13.....	70	41
4.....	86	47	9.....	79	61	14.....	71	50
5.....	86	58	10.....	87	59	15.....	78	35
Av..	81.6	48.5	.....	77.0	56.4	.....	68.8	41.0

The table shows that in every lot the head frequency is distinctly higher when posterior section of the nerve cords is made under anesthesia (Expts. *G*, *J*, *L*) than when section of both nerve cords (Expts. *H*, *K*) or one nerve cord (Expt. *M*) is made in water after apparent recovery.

In the second set of experiments the procedure was the same except that the series of partial sections under anesthesia was made anteriorly instead of posteriorly (Figs. 18–23). The data given in Table V show that in every lot head frequency is higher when anterior section of the nerve cords is made under anesthesia (Expts. N, Q, S) than when section of both nerve cords (Expts. *P*, *R*) or of one cord (Expt. *T*) is made in water after apparent recovery.

In all cases of section of the nerve cords under anesthesia, whether at the posterior or the anterior end of the piece, the cut ends of the

cords are directly exposed to the anesthetic for 10 minutes after section and presumably are more deeply anesthetized than the parts not so exposed. As shown in Series II, 30 minutes' delay of anterior or posterior section without anesthesia does not alter head frequency appreciably; but in the present series, head frequency is markedly increased by either anterior or posterior section of the nerve cords under anesthesia with 10 minutes' exposure to the anesthetic after section and 20 minutes in water, i.e., 30 minutes' delay of other parts of section. Evidently, direct exposure of the nerve cords to the

TABLE V

EFFECT ON HEAD FREQUENCY OF PARTIAL ANTERIOR SECTION UNDER ANESTHESIA

Each lot consists of 40 pieces, 20 for each of the two experiments of the lot.

Lot	Experiments		Lot	Experiments		Lot	Experiments	
	N	P		Q	R		S	T
1.....	75	66	5.....	77	65	9.....	69	56
2.....	86	61	6.....	82	58	10.....	75	61
3.....	87	63	7.....	87	69	11.....	79	57
4.....	92	64	8.....	88	60	12.....	82	64
Av..	85.0	63.5	.....	83.5	63.0	.....	76.2	59.5

anesthetic by section is more effective in blocking the factor inhibiting head development even after 20 minutes' recovery than direct exposure of other parts by section.

Moreover, the data of Series III above show that direct exposure of either anterior or posterior cut surface to the anesthetic is more effective in blocking the inhibiting factor and increasing head frequency than the same period of anesthesia of intact animals. In both Series III and the present series, the effect on head frequency is essentially the same whether direct exposure to the anesthetic is at the posterior end of the piece. It seems evident from both Series III and Series V that, with direct exposure of the whole cut surface or of the nerve cords to the anesthetic at either anterior or posterior end of the piece, the effect persists long enough to determine a marked increase in head frequency.

The experiments in which a half posterior or anterior half-section,

including one nerve cord, is made under anesthesia (Expt. *M*, Table IV, Fig. 17; *T*, Table V, Fig. 23) show a head frequency as low as, or lower than, those in which both nerve cords are cut in water after anesthesia (*H* and *K*, Table IV, Figs. 13, 15; *P* and *R*, Table V, Figs. 19, 21). It was shown, however, in an earlier paper (Child and Watanabe, 1935, pp. 21–26) that in *Euplanaria dorotocephala* posterior half-section including one nerve cord does not decrease head frequency appreciably; and *F*, Table III (Fig. 11), gives the same experiment with the same result for *E. maculata*. Apparently head development is strongly inhibited only when both nerve cords are cut, even though the inhibiting factor may be more or less blocked by anesthesia in one cord. In the earlier paper it was suggested that the high head frequency after posterior section of one cord might be due to the formation of a complete head by the cells of the uncut side. The later experiments of Series III and V indicate, however, that the factor inhibiting head development can be more or less eliminated on one side by anesthesia and a low head frequency still result from later posterior section of the other cord. Perhaps the effect of posterior section is less in a piece of cord when connected by transverse commissures with a cord not sectioned posteriorly, but only further experiment can throw light on this point.

In the experiments of Series V pieces died, evidently from exposure to chloretone, as follows: in Experiment *G*, 1; in *H*, 2; in *K*, 1; in *M*, 1; in *N*, 4; in *Q*, 4; in *S*, 3; and in *T*, 1. The highest death-rate in Experiments *N* and *Q*, with 80 pieces each, was 5 per cent. Bipolar forms occurred in all experiments except *M*, the total number being 46 among 1,080 pieces. The highest percentages of bipolar forms are 9 in Experiment *J* and 8.75 in Experiment *Q;* the next highest is 3.75 in experiment *P*. The relatively high percentages in *J* and *Q*, in which both nerve cords and one lateral region are cut either posteriorly (*J*) or anteriorly (*Q*) under anesthesia and isolation of the piece is completed in water, suggests a relation between this type of experiment and bipolar frequency; but the bipolar frequencies are too low to permit a definite conclusion.

DISCUSSION

Head frequency in relation to length of piece and body-level.—As Table I and Figure 1 show, head frequency in *Euplanaria maculata*

decreases from anterior levels to the middle region and increases again in the posterior zooid region; and the decrease and increase become greater as length of piece decreases. The head-frequency gradient is a double or U-shaped gradient which increases in steepness with decreasing length of piece and is similar to the head-frequency gradients in other species of *Euplanaria* examined, which have a posterior zooid region, viz., *E. lata* (Šivickis, 1923, Fig. 21), *E. dorotocephala* (Child and Watanabe, 1935, Fig. 1) and *E. novangliae* (Child, unpublished data). In *E. maculata* the head-frequency gradients are less steep for given fractions of body length than in *E. dorotocephala;* and if Šivickis' data graphed by a different formula from that used in this paper are recalculated, the gradients in *E. lata* are somewhat less steep than in *E. maculata*, and the lowest frequencies in 1/6 and 1/8 pieces of *E. lata* are farther posterior than in *E. maculata*. This difference is in accord with the fact that in *E. lata* the zone of fission is farther posterior than in *E. maculata*.

Bipolar frequencies in pieces of corresponding fractional length also differ in the three species, under normal conditions. In *E. lata*, according to Šivickis, bipolar forms appear very rarely in 1/6 pieces and somewhat more frequently in 1/8 pieces (maximum 1.6 per cent); but in *E. maculata* 6 per cent of pieces approximately 1/8 are bipolar. In *E. lata* this percentage of bipolar forms is reached only in 1/16 pieces from the posterior region. In *E. dorotocephala* bipolar forms usually occur rarely in normal environment, even in 1/16 pieces of large animals; but their frequency can be greatly increased experimentally (Rustia, 1925). In certain stocks of *E. dorotocephala* collected from one locality in winter and early spring of 1934 an abnormally high head frequency (almost 100 per cent normal heads from 1/8 pieces of all body levels) and a high bipolar frequency were observed. The animals of this stock were apparently, for some unknown reason, not in normal condition. Many individuals died in the laboratory environment in which they usually live indefinitely.

In general it is evident that in all species studied bipolar frequency increases with decreasing length of piece and is higher in the posterior zooid region than anteriorly. The frequency of bipolar forms in the experiments on delayed section is too low to give any

definite evidence of relation to particular types of experiment, except for the possible relation noted on p. 388.

Experimental analysis of factors determining head frequency.—Earlier work has made it evident that two antagonistic factors are concerned in the reconstitution of planarian heads—the one concerned with the activation, redifferentiation, and growth of the cells near the cut surface which give rise to the head; the other, a factor of some sort inhibiting these reconstitutional activities in greater or less degree. Head frequency depends on the relation between these two factors. The positive head-determining factor has been considered to be the physiological condition or activity of the cells which give rise to the head and is related to body-level, that is, to the level of the gradient at which section occurs. The negative inhibitory factor was regarded as related to the physiological condition or metabolic activity in the remainder of the piece resulting from stimulation by the posterior cut. The more intense this factor in relation to the intensity of the determining factor, the greater the degree of inhibition of head development; and vice versa (Child, 1914*a*, 1914*b*, 1916, 1920, 1921; Behre, 1918; Buchanan, 1922; Šivickis, 1923). Buchanan (1922) suggested, and more recent work (Child and Watanabe, 1935) has supported, the view that the inhibiting factor is largely or wholly nervous in character. The experiments on delay of posterior and anterior complete section and posterior and anterior section of the nerve cords with and without anesthesia are attempts at the further analysis of the problem of head frequency, and particularly of the inhibiting factor.

The experiments on delay of posterior or anterior section in normal environment give results essentially similar to those obtained with *Euplanaria dorotocephala* (Child and Watanabe, 1935), except that in the latter species delay of section for a much longer time than in *E. maculata* is necessary for the attainment of maximal head frequency. This difference in the species is to be expected from the fact that in *E. dorotocephala* the inhibiting factor is more effective than in *E. maculata*. The results of these experiments show that if posterior section is delayed for 6 hours or more after anterior section, the inhibiting factor is wholly or almost ineffective.

With delay of anterior section, however, the inhibiting factor ceases to be effective in inhibiting head development only after 36

hours or more. With 12 hours' delay of anterior section the inhibiting factor is more effective than when anterior and posterior section are made at the same time (Fig. 3), and a similar condition occurs in *E. dorotocephala* (Child and Watanabe, 1935, Fig. 5). This fact, together with the long delay of anterior section necessary for attainment of maximal head frequency, suggests that the cell activity following the posterior section, as well as the section itself, is concerned in giving rise to the factor inhibiting head development, and that this factor increases in effectiveness for some time after section.

The experiments of Series III (Table II), in which one section is made under anesthesia and the other after more or less complete recovery, show that when either posterior or anterior section is made under anesthesia the inhibiting factor is much less effective and head frequency is higher than in the controls. Series IV and V (Tables III–V) show further that posterior section of the nerve cords is much more effective in inhibiting head development than section of other regions, and that either posterior or anterior section of the nerve cords under anesthesia eliminates or blocks the inhibiting factor to a much greater degree than section of other parts under anesthesia. These results give further evidence in support of the conclusion that the factor inhibiting head development is primarily nervous and that the nerve cords are its chief path, though other parts of the nervous system may also play some part in its transmission.

In certain experiments of Series V the nerve cords are directly exposed to the anesthetic by section either at the anterior or the posterior end of the piece, and in both cases head frequency is higher than in the experiments without direct exposure of the nerve cords to the anesthetic. It seems evident that such direct exposure of the cut surface of the nerve cords produces a greater and more persistent anesthetic effect than the indirect exposure through other tissues, or that it may bring about more or less irreversible injury near the cut surface. Whichever the case, it is effective in blocking the inhibiting factor even after 20 minutes in water—and how much longer is not known. The block is probably near the anterior or posterior cut surface according as anterior or posterior section is made in the anesthetic.

The fact that the effectiveness of the inhibiting factor decreases

with increasing distance of posterior from anterior cut surface suggests that it undergoes a decrement and that the decrement differs in steepness in different regions of the body, but another possible interpretation of the facts may be noted. Some of the experiments on partial section suggest the possibility that the effect of posterior section increases in intensity as the length of nerve cord isolated decreases; and that even when a short piece of one nerve cord is isolated from the rest of that cord, but remains in connection with the other cord either through transverse commissures or otherwise, the inhibiting effect on head development is less than when it is completely isolated. According to this suggestion, the fact that the inhibiting factor resulting from posterior section is effective over a greater length of piece in the posterior than in the anterior region of the anterior zooid and in the posterior zooid region must mean that different regions of the nerve cords are affected to different degrees by isolation. However, the data at hand do not permit us to determine whether the relation between head frequency, length of piece, and body-level is due to a decrement in the anterior direction of the effect of posterior section or to an increasing intensity of effect with decrease in length of piece.

In conclusion, it is evident that the data presented in this paper confirm the earlier view that head frequency in *Euplanaria* involves two factors—one, the determining factor, the condition and activity of the cells which give rise to the head; the other, the inhibiting factor, the excitation in the piece posterior to the region of head development. These data also give various lines of evidence in support of the conclusion that the inhibiting factor is primarily nervous in character and that the nerve cords are its chief path of transmission.

In the light of the evidence at hand the situation as regards reconstitution of the head in pieces of *Euplanaria* appears to be somewhat as follows: Any level of the postcephalic region is potentially a head. In the intact animal it does not become a head because it is prevented from doing so by the physiological action upon it of other parts, chiefly of parts anterior to it but also to some extent of parts posterior to it. In consequence of these relations to other parts, it maintains the characteristics of a particular body-level. When parts

anterior to this level are removed, it is no longer subordinated to them, and head development occurs unless the physiological factor from more posterior levels is sufficiently effective to inhibit the activation and dedifferentiation of the cells concerned in head formation. In the absence of a posterior cut surface this factor is not effective in inhibiting head development. The posterior section evidently increases its effectiveness, but its effectiveness decreases with increasing distance of the posterior cut surface from the anterior. If the posterior cut surface and the head-forming region are within a certain distance of each other which differs according to body-level, the excitation, primarily nervous in character, which results from posterior section becomes more or less effective in inhibiting the changes leading to head formation in *Euplanaria*.

In short, head development occurs when the cells concerned are independent of other regions of the body and is inhibited to the degree to which they become subordinate to other regions, usually to more posterior regions. This conception and the experimental data on which it is based do not support the view that the planarian head is reconstituted from undifferentiated "formative cells." The experimental evidence indicates that the cells concerned in head formation are affected by correlative factors originating in other parts of the body. If they are so affected, they are presumably more or less differentiated; and in order to give rise to a head, they must become independent of correlative factors and undergo dedifferentiation.

SUMMARY

1. Head frequency in *Euplanaria maculata* shows a relation to length of piece and body-level similar to that in other species of *Euplanaria* studied, but the steepness of the gradient for a given fractional length of piece is less than in *E. dorotocephala* and somewhat greater than in *E. lata*.

2. Delay of either posterior or anterior section increases head frequency in short pieces (1.5 mm. long) of full-grown animals. Six hours' delay of posterior section and 36 hours' delay of anterior section at temperatures near 20° C. gives maximal, or almost maximal, head frequency.

3. In short pieces with low head frequency when anterior and posterior section are made at the same time in water, head frequency is increased by making either anterior or posterior section under anesthesia and the other section 30 minutes later after 20 minutes in water.

4. Posterior section of the nerve cords with delayed section of the other parts is more effective in inhibiting head development than posterior section of other regions.

5. Section of the nerve cords either at the posterior or anterior end of the piece under chloretone anesthesia and in the chloretone solution is much more effective in blocking the factor inhibiting head development, and so increasing head frequency, than section of other parts in the anesthetic.

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